Advances in CLINICAL CHEMISTRY

VOLUME 45

Edited by

Gregory S. Makowski

ADVANCES IN CLINICAL CHEMISTRY

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Advances in CLINICAL CHEMISTRY

Edited by

GREGORY S. MAKOWSKI

Department of Pathology and Laboratory Medicine
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VOLUME 45

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PREFACE

I am pleased to present volume forty-five of *Advances in Clinical Chemistry* series for the year 2008.

In the first chapter of this volume, the role of gonadal inhibins as diagnostic markers in human pathophysiology is explored. This chapter highlights the development of specific immunoassays that helped to elucidate this important hormone in biochemical control systems. The clinical utility of plasminogen activators and their inhibitors is reviewed with respect to their pleiotropic roles in neoplastic growth including fibrinolysis, cell migration, and angiogenesis. Special emphasis is put on their role in breast cancer. The continuing evolution of multiple analyte profiling is also explored in this volume with respect to its useful and widespread application in complex biological systems. An interesting chapter evaluates the use of immune biomarkers in clinical trials. This chapter includes multiplex, genomic and proteomic technology for cytokine analysis. Biochemical indices of brain function are explored through the use of dietary modulation in another chapter that brings together biochemical and physiologic aspects of mental health. The role of gonadotropins in Alzheimer's disease is explored in a chapter that comprehensively reviews etiologic, epidemiologic, and pathologic evidences in the literature. The use of gonadotropins as specific therapeutic targets is also investigated. A chapter on kynurenines explores the important biochemical and pathophysiologic roles of this key enzymatic pathway in immunosuppression. Lastly, the prognostic significance of tumor-associated macrophages as a human defense mechanism is reviewed with respect to their pro- or antitumor activity.

I extend my appreciation to each contributor of volume forty-five and also thank colleagues who contributed to the peer review process. I extend my most sincere thanks to my editorial liaison at Elsevier, Ms. Pat Gonzalez, for her advice and continued support.

I hope the first volume of 2008 will be enjoyed by our readership. As always, I warmly invite comments on past volumes and suggestions for future review articles for the *Advances in Clinical Chemistry* series.

In keeping with the tradition of the series, I would like to dedicate volume forty-five to my brother Keith.

GREGORY S. MAKOWSKI

INHIBINS AS DIAGNOSTIC MARKERS IN HUMAN REPRODUCTION

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1. Abstract

Over the past 75 years, many publications have focused on measurement of inhibin concentration and/or activity in biological samples in order to

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understand its role in physiology and disease. This chapter highlights the accomplishments within this area of research over the past decade including development of specific inhibin assays. Inhibin A is a marker of dominant follicle and corpus luteum activity and decreases in polycystic ovary syndrome (PCOS). Inhibin A increases in gestational diseases such as pre-eclampsia and fetal Down's syndrome, and this increase in inhibin A improves early diagnosis of both conditions. The measurement of inhibin A in women with threatened abortion provides useful information about the likelihood of pregnancy loss. Inhibin B increases markedly in women with granulosa cell tumor and appears closely related to gametogenesis in men, that is, reflecting Sertoli cell activity. On the contrary, Inhibin B decreases in women with declining ovarian function and correlates with female response to ovulation induction. This review evaluates the biochemical significance of inhibins including their use in clinical practice.

2. History and Background

Inhibin is a gonadal hormone that exerts specific negative feedback on pituitary secretion of follicle stimulating hormone (FSH). The existence of inhibin was postulated in 1923 by Mottram and Cramer who observed hypertrophy of pituitary cells after irradiation of the testis of adult rats [1]. McCullagh [2] subsequently investigated this phenomenon by injecting a water-soluble extract from bovine testes and found that it could inhibit hypertrophy of the pituitary and thus named the active, water-soluble principle "inhibin" [2].

The concept of inhibin was refined after the discovery of two separate pituitary hormones, FSH and luteinizing hormone (LH), which together regulate the gonadal function. Klinefelter *et al*. [3] observed that serum levels of FSH were elevated in oligo- or azoospermic men. Heller and Nelson [4] suggested that the increased serum levels of FSH were caused by a lack of utilization of FSH by the defective seminiferous tubules. Some groups [5, 6] showed that the injection of water-soluble testicular extracts into rats could suppress peripheral FSH levels. de Jong and Sharpe [7] also found FSH-suppressing activity in ovarian follicular fluid. Little, however, was understood about the mechanism of action of inhibin on pituitary cells until Franchimont *et al.* [8] demonstrated that the addition of inhibin-containing preparations to pituitary cells caused an increase in the secretion of cGMP and a decrease in cAMP secretion. This finding was then followed by Jenner [9] who speculated that the decrease in cAMP in incubated hemipituitaries is followed by a decrease in the synthesis of FSH.

The combination of an FSH radioimmunoassay (RIA) and an *in vitro* primary pituitary cell culture led to the purification of inhibin from bovine [10] and porcine [11–13] follicular fluids. Cloning established the protein sequence of inhibin as a 32-kDa dimeric glycosylated molecule consisting of an α and a β subunit. It soon became clear that there were two forms of inhibin. Each contains a common α subunit associated with either β_A (inhibin A) or β_B (inhibin B) subunits that also possess a high degree of homology (Table 1) [12]. Inhibin A and inhibin B were found to be equipotent in the rat pituitary cell bioassay [14], but in the sheep pituitary cell bioassay [10], inhibin A was much more potent than inhibin B. During the purification in bovine, it became clear that there was a large amount of free α chain inhibin that had no bioactivity [10] and could potentially interfere with quantitative assay unless the models were specifically configured to ignore its presence. Initially, inhibin was identifiable as a distinct entity only by bioassays [15–18]. These, of course, were of limited specificity, and generally unsuitable for use with complex body fluids. The development of highly specific and sensitive immunoassays proved essential in advancing the study of inhibin in physiologic as well as pathophysiologic conditions.

The original Monash RIA [19] was widely used in physiological studies before it was realized that it was detecting mostly free α subunits rather than dimeric bioactive inhibin forms in human body fluids [20]. The breakthrough in our understanding of the biology of inhibin came when Groome and collaborators established a specific immunoassay for the measurement of dimeric inhibin A in the menstrual cycle [21]. An assay for measurement of dimeric inhibin B was also subsequently developed by this group [22]. Both assays have been found to be useful for many clinical applications, some of which are detailed below.

TABLE 1
Schematic Representation of Precursor and Mature Forms of Inhibin A and Inhibin B and Mature Forms of Activin

		Structure	Molecular weight (kDa)
α-Subunit precursor	α Chain	pro + αN + αC	55
	pro-αC	pro + αC	26
β-Subunit precursor	β_A Chain	proβ_A + β_A	58
	β_B Chain	proβ_B + β_B	58
Mature forms	Inhibin A	$\alpha + \beta_A$	32
	Inhibin B	$\alpha + \beta_B$	32
	Activin A	$\beta_A + \beta_A$	28
	Activin B	$\beta_B + \beta_B$	28
	Activin AB	$\beta_A + \beta_B$	28

During the purification of inhibin from porcine follicular fluid, two research groups isolated a protein that displayed FSH-releasing activity from the gonadotrophs *in vitro* [23–26]. This protein was termed "activin," signifying its antagonistic effect to inhibin. The discovery of new genes related to the inhibin β subunit through the use of degenerate polymerase chain reaction (PCR) cloning methods further complicated the inhibinstory. There are now three known types of bioactive activins: activin A, activin AB, and activin B (Table 1). Three recently identified activins (C, D, and E) are not known to have any bioactivity and have a relatively restrictive tissue distribution. Activin has proven relevant to diverse research fields, including cell biology, developmental biology, and endocrinology. Activin bioassays have the same problems as inhibin, that is, a lack of specificity.

During purification of inhibin, another weak inhibitor of FSH secretion was identified, named follistatin, which was purified from both porcine [27] and bovine follicular fluid. It was soon realized that follistatin acted by binding to activin and thus neutralizing its actions. The protein is expressed abundantly in the granulosa cells of healthy antral follicles. Shimonaka *et al.* [27] demonstrated by double ligand blotting technique that activin A has two binding sites for follistatin, whereas inhibin A has one. From this, it can be shown that follistatin interacts with inhibin A and activin A through the common β subunit. Follistatin has the ability to neutralize activin-induced FSH release from pituitary cell cultures but this bioassay, like those for inhibins and activins, is susceptible to interference [28]. A number of immunoassays for total follistatin have now been established [29].

Another activin-binding protein of 70 amino acids (aa) has been identified as a follistatin-related gene (FLRG) product, based upon its primary sequence homology to follistatin and its modular architecture, which is remarkably similar to that of follistatin [30, 31]. Like follistatin, FLRG also interacts physically with activin A. This interaction prevents binding to activin receptors (ActRs) [31, 32], thus suggesting a regulatory role on activin-mediated cellular processes.

Both activin and inhibin have been classified as members of the transforming growth factor-β (TGF-β) superfamily [23, 25, 28, 32–34]. They are synthesized as large precursors containing a signal sequence, a pro-domain of variable size, and a mature C-terminal segment that ranges from 110 to 140 aa in length. Within the mature segment, there are seven cysteine residues that are invariant across the superfamily.

Inhibin A consists of an α subunit disulfide linked to a β_A [$\alpha + \beta_A$] subunit, and inhibin B consists of an α subunit and a β_B [$\alpha + \beta_B$] subunit (Table 1). Activin A contains two β_A subunits, activin B has two β_B subunits, and activin AB has one β_A and one β_B subunit (Table 1). cDNAs for the α, β_A,

and β_B subunits have been cloned from porcine, human [9, 32, 35], bovine [36, 37], rat [38], mouse [39], and *Xenopus* [40] cDNA libraries.

In humans, the α and β_B subunits are encoded by individual genes and have been assigned to chromosome 2. β_A subunit gene has been assigned to chromosome 7 [35, 36]. The subunits are produced as large precursors containing an N-terminal signal peptide targeting the polypeptides to the endoplasmic reticulum (ER), a pro-region, and a mature C-terminal domain, which is found in the bioactive dimers [41]. The pro-regions, which are needed for correct folding and/or dimerization of the subunits, are cleaved from the mature bioactive inhibin and activin proteins before secretion [42]. However, there is an exception identified in the 56 kDa bovine inhibin [37] and the 65 and 55 kDa human inhibin [14] in which either the fully or partially processed pro-regions are cleaved after secretion. Recombinant inhibins of 55–65 kDa show bioactivity comparable to that of the 32 kDa inhibin [43], indicating that the cleavage of the pro-region is not required for bioactivity.

The α subunit is unique to inhibin molecules. It is synthesized as a 53 kDa, 366 aa (depending on species) precursor containing an 18 aa signal sequence, a 43 aa pro-region, also called a pro-α, and a 305 aa mature form. The mature form is itself divided into a 171 aa N-terminal segment (αN) and a 133–134 aa C-terminal segment (αC). Unlike the β chain, however, the mature (αC) fragment has sites for glycosylation, and usually shows mono- and diglycosylation [41].

The genomic organization of the human [35], rat [38], mouse [39], and bovine [37] α subunit gene has been determined. In these species, only one inhibin α subunit gene has been found, containing two exons and a single intron of about 2 kilobases (kb) located in the sequence encoding the pro-region.

The mammalian mRNA for both β subunits encodes ~400 aa polypeptide chains, which, like the α subunit, includes a signal sequence, a pro-region and a C-terminal mature region (Table 1). The mature β_A subunit has 116 aa, and the β_B has 115 aa. These proteins are characterized by the cleavage of the mature region from the C-terminal of the large precursor, and by the presence of nine cysteine residues in the mature region. These are involved in disulfide linkage; eight of these cysteine residues are involved in intra-subunit disulfide linkage, and the remaining one is used for the inter-subunit disulfide linkage. Recent studies show three new activin β chains [44–46] named activin β_C, β_D, and β_E subunits. The β_C subunit was cloned from a human liver cDNA library and shows about 50% homology with the β_A and the β_B subunit. The β_D was cloned from *Xenopus* liver cDNA and shows 60% homology with β_C [45], while the β_E subunit was cloned from mouse liver [46] and shows 60% homology with β_D and β_C.

3. Inhibins in Women's Reproductive Function

3.1. Effects of Inhibin on Pituitary FSH Secretion

It is clear from the preceding section that inhibin acts as an endocrine signal generated from the gonads to provide a monitor of gonadal activity to control pituitary FSH secretion. In addition, the α and the β_B subunits, but not the β_A subunit, were found to be localized in the cytoplasm of FSH- and LH-immunoreactive gonadotropes. This suggests that the gonadotropes are sources, as well as targets, of inhibin-related peptides, where their expression is modulated by ovarian factors [47, 48].

Passive immunoneutralization of inhibin stimulated FSHβ mRNA levels and FSH secretion in rats [49], whereas the addition of inhibin to cultured pituitary cells suppressed FSHβ mRNA levels [50]. Pituitary activin has been demonstrated to play an autocrine role in maintaining FSHβ mRNA levels and FSH secretion *in vitro* [51], and exogenous activin stimulated FSHβ mRNA levels within 2 hours in cultured cells [49]. Activin has also been shown to increase GnRH receptor expression through a specific transcriptional activin response element [52]. Thus, activin could influence FSH synthesis indirectly through enhancement of GnRH signaling. Furthermore, follistatin can block GnRH-induced FSH secretion *in vitro*, suggesting that some of the actions of GnRH on FSH may involve local activin stimulation [53].

Immunoneutralization of activin B in primary pituitary cell cultures resulted in suppression of FSH secretion, while follistatin has recently been shown to exert the same effect *in vivo* [54] and *in vitro* [55]. In rats, inhibin B is synthesized in the pituitary and both α and β_B subunits and mRNA are present in rat gonadotropes [56]. Thus, all the components of an activin B driver, follistatin modulator, and inhibin B inhibitor system are present within the pituitary, allowing autocrine control of FSH synthesis and secretion.

The past years have seen rapid changes and increases in the use of transgenic mouse technology for studying mammalian development [57–60]. Three mouse models in which the regulation of FSH expression by inhibins and activins has been evaluated have been developed by Matzuk *et al.* [60]. Mice lacking the inhibin α subunit demonstrate increased FSH concentrations, and this confirms the known function of inhibins [60].

Mice deficient in activin receptor type II have markedly suppressed pituitary and serum concentrations of FSH. Thus, this receptor appears to be the major pituitary receptor through which activins regulate FSH synthesis and secretion. In addition, the suppressed FSH synthesis in the activin receptor type II-deficient mice leads to hypogonadal phenotype in both sexes.

In general, although effects of the gonadal peptides could be measured in specific biological experiments, the overall regulatory role appeared to be less powerful than that of the sex steroids and GnRH. The ability, however, of activin and inhibin preferentially to modulate FSH provides an attractive basis to explain some of the divergent secretory profiles of LH and FSH during the reproductive cycle.

3.2. Inhibin During the Menstrual Cycle

The ovary is responsible for the circulation of inhibin A and inhibin B in healthy women [61]. Inhibin is secreted by the granulosa cells of the developing follicles in the corpus luteum in response to gonadotropins and various other factors. These include insulin-like growth factor (IGF), TGF-β, and activin, while FSH-induced inhibin secretion is suppressed by epidermal growth factor (EGF), TGF-β, and follistatin [13, 62, 63].

In the follicular phase of the menstrual cycle, both ovaries secrete similar amounts of inhibin. In the luteal phase, most of the inhibin is secreted by the corpus luteum [61]. Dimeric inhibins were assayed by Groome *et al.* [21] for the first time in healthy women throughout the menstrual cycle. The inhibin A plasma concentration remains low ($<$10 pg/ml) during the early follicular phase, then shows a small midfollicular phase peak. The concentration rises rapidly with ovulation to a maximum of $\sim$60 pg/ml. The inhibin A concentration falls synchronously with the drop in progesterone during the late luteal phase.

Inhibin B is different in that the plasma concentration is high in the early follicular phase and falls in the late follicular phase during the days before ovulation (Fig. 1). There is no increase in its concentration in the midcycle LH peak. Two days later, there is a short-lived peak of the hormone concentration before the concentration drops to a low level by the middle of the luteal phase. The plasma inhibin B concentration subsequently remains low during the luteal phase [22].

The expression of inhibin α and β_A subunit mRNAs in granulosa cells increases with follicular development [64]. Dimeric inhibin A levels in the follicular fluid increase as the preovulatory follicles mature [21], coincident with the late follicular phase raise in peripheral serum inhibin A concentrations. Conversely, the expression of β_B subunit mRNA in granulosa cells and levels of inhibin B in follicular fluid are highest in immature follicles present during the ovary follicular phase of the menstrual cycle, when peripheral serum concentrations of inhibin B are also increased [23, 65, 66]. Hence, the data demonstrate a clear inverse correlation between similar plasma levels of inhibin B and the corresponding FSH concentration. This suggests that the developing follicles secrete inhibin B, suppressing FSH secretion in the follicular phase, whereas inhibin A is secreted from the luteal cells, which control FSH in the luteal phase.

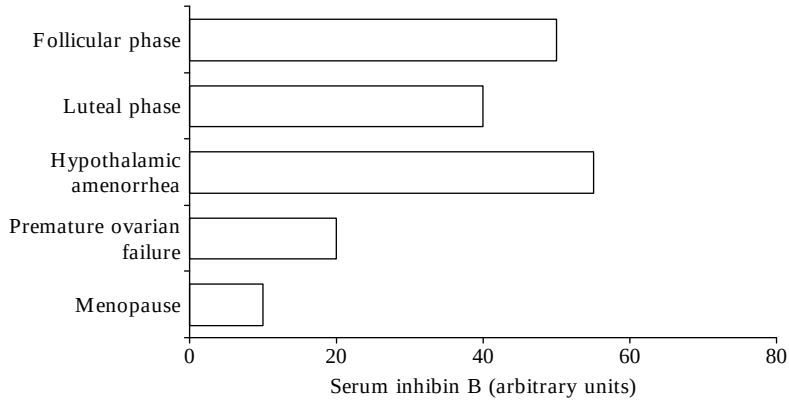

FIG. 1. Serum inhibin B concentration in women's reproductive function.

3.3. CONTROL OF INHIBIN B BY FSH IN GNRH-DEFICIENT WOMEN

It is difficult to distinguish the relationship between inhibin and FSH, as gonadotrophin stimulation is known to promote inhibin secretion, which, in turn, exerts a negative feedback on FSH. The study conducted by Welt *et al.* [67] used a model of GnRH-deficient women to explore the relationship between FSH and inhibin B secretion during the luteal-follicular transition by manipulating FSH levels via changes in the frequency of exogenous GnRH administration. The GnRH pulse frequency was either increased from every 4 hours in the late luteal phase to every 90 min on the day of menses to mimic normal cycling women or kept constant at a late luteal phase frequency through the first 6 days of the subsequent cycle. The slower rate of rise in FSH observed in the group in which the GnRH pulse frequency was maintained at the slow luteal phase frequency was associated with significantly lower inhibin B levels in the early follicular phase. Hall *et al.* [68] suggest that there may be a critical rate of rise in FSH required for inhibin B stimulation. In these studies, the difference in the inhibin B responses to varying FSH levels was evident before changes were apparent in estradiol levels, indicating that inhibin B may be an important early prognostic indicator of follicular response during ovulation induction therapy [67, 69, 70].

3.4. INHIBINS AND ACTIVIN A IN OVULATION INDUCTION

Several studies have reported an increase in total immunoreactive inhibin (ir-inhibin) levels after the administration of exogenous gonadotropins for ovulation induction [71]. These studies have noted a correlation between the

inhibin response and the number of follicles stimulated as well as a decline in the inhibin response with increasing reproductive age. More detailed information has come from studies employing pituitary downregulation with GnRH analogue before stimulation with recombinant FSH for *in vitro* fertilization (IVF), as this regimen creates a hormonal environment that allows the impact of high doses of FSH on ovarian inhibin secretion to be selectively elucidated.

In a study of women with normal ovarian function participating in such an IVF treatment protocol, Lockwood *et al.* [70] demonstrated that both inhibin A and inhibin B were significantly suppressed in association with pituitary desensitization, whereas levels of pro-αC and activin A were largely unaltered. Levels of both inhibin A and inhibin B rose markedly during stimulation with FSH. The positive correlation observed between inhibin A levels in the late follicular phase and the number of follicles larger than 10 mm suggests that inhibin A may be useful as a marker of dominant follicle development in IVF. These data indicate that ovarian production of dimeric inhibin A and inhibin B in women is gonadotropin dependent, whereas the regulation of pro-αC may have a significant gonadotrophin-independent component [72, 73]. Casper *et al.* [74] also investigated the concentrations of inhibin/activin in women undergoing stimulation with recombinant FSH for IVF treatment. There was no change in activin A levels with rFSH stimulation, which is in agreement with Lockwood *et al.* [70]. In women with sonography-detected leading follicle >17 mm in diameter, levels of inhibin A, pro-αC, and estradiol increased significantly, but not that of inhibin B. In patients without adequate follicle development during FSH stimulation, serum levels of inhibins remained low and did not significantly deviate, indicating a possible marker of follicular development.

Other potential applications of inhibin assays in relation to IVF have been proposed. Eldar-Geva *et al.* [75] found that serum inhibin B concentrations measured during the early stage after administration of fixed-dose recombinant human FSH treatment provided an early predictor of the number of recruited follicles. Lindheim *et al.* [76] reported that the measurement of inhibin A may be used to identify the women undergoing natural-cycle embryo transfer who should be prepared for egg retrieval. Lockwood *et al.* [70] found initially a rise in maternal serum inhibin A levels and then a rapid decline in pregnancies in embryonic failure, indicating a possible clinical application in the diagnosis of failed pregnancies.

3.5. Premature Ovarian Failure

Inhibin A and inhibin B are dramatically reduced in women with premature ovarian failure (POF) [77]. Serum levels of both inhibins are as low as in normal postmenopausal women matched for time elapsed since last menstrual period,

and do not correlate with patient age, length of amenorrhoea, or serum gonadotropin levels [77]. In addition, circulating levels of inhibin A, inhibin B, and pro-αC are reduced after oophorectomy. Women with amenorrhoea induced by GnRH analogue treatment or by antineoplastic chemotherapy still produce inhibin A and pro-αC [73]. This probably reflects a residual ovarian function and hormone synthesis.

Welt *et al.* [78] demonstrated high inhibin B serum concentrations in women with presumptive autoimmune oophoritis, who developed multiple follicles with low to undetectable estradiol levels in circulation. These data may provide further evidence for an important role of inhibin B and inhibin A in the negative feedback control of FSH. In addition, the normal inhibin A and inhibin B production in the absence of estradiol precursors and estradiol provide insight into the selective dysfunction of the theca cells in autoimmune oophoritis.

3.6. Inhibin and Activin in Polycystic Ovary Syndrome

One of the most common causes of female infertility is the PCOS, affecting approximately 6-8% of women of reproductive age [79]. PCOS is characterized by an excessive number of small antral follicles in the ovaries that fail to produce a dominant follicle on a regular basis, and by dissociation in LH and FSH release [79].

Inhibin A and inhibin B are produced by the granulosa cells of human ovary. Since the α subunit is also expressed by the theca interna cells in addition to the granulosa cells, the levels of α subunit mRNA in the ovary are higher than those of β subunit mRNA [80].

Levels of inhibin B have been found to be either increased [81] or unchanged [82–85] in women with PCOS, and this disagreement might be due to the large range of inhibin B levels seen in PCOS and also to the confounding effect of obesity—an independent factor that reduces inhibin B concentration [84–85]. In addition, women with clomiphene-resistant PCOS show no pattern of regular inhibin B pulsatility when compared to normal individuals [81].

We have also observed that inhibin A concentrations are consistently lower in women with PCOS (Tsigkou *et al.*, unpublished data), confirming previous findings in a large, well-characterized sample of women with PCOS [82]. The low circulating levels of inhibin A and the lack of increase in inhibin B levels despite the numerous antral follicles in women with PCOS converge to the same pathophysiological mechanism, which is the insufficient production of dimeric inhibins by the many small follicles that fail to reach dominance and ovulation [86].

Magoffin and Jakimiuk [86] investigated follicular fluid concentrations of inhibin A, inhibin B, and activin A in women with regular cycles and in

women with PCOS. In both control ovaries and polycystic ovaries, they found that concentrations of inhibin B were approximately tenfold higher than those of activin A. There was no difference in activin A concentration between PCOS and control follicles. In control ovaries, the inhibin A and inhibin B concentrations in dominant follicles were significantly higher than in cohort follicles, while inhibin A concentrations were lower in PCOS follicles than in normal cohort follicles. However, there was no difference in inhibin B concentrations between PCOS follicles and normal cohort follicles [86].

3.7. Reproductive Aging

One of the areas of increasing interest in female reproductive endocrinology is the hormonal environment during the early phase of reproductive aging, a time when there is a selective rise in FSH levels. Given that no significant changes have been observed in ovarian steroid secretion at this time, it has been postulated that early decreases in ovarian inhibin secretion might account for the monotropic FSH rise seen in older ovulatory women [87].

Initial studies measuring total ir-inhibin demonstrated that inhibin levels were decreased in perimenopausal women and were undetectable in postmenopausal women [88]. An increase in activin A secretion was also observed during reproductive aging in women [89]. In a correlative study employing dimer-specific ELISAs, Klein *et al.* [90] reported that higher early follicular phase FSH levels in a group of older, ovulatory women (aged 40–45 years) were associated with significantly lower mean inhibin B concentrations compared to those in younger cycling controls [90]. Burger *et al.* [88] identified a fall in inhibin B circulating during the follicular phase and no changes in estradiol and inhibin A as the most significant endocrine observation of reproductive aging. These observations were also confirmed by Welt *et al.* [69].

Danforth *et al.* [91] reported a fall in circulating inhibin A during the luteal phase of women undergoing the menopause transition [91]. The decrease in inhibin B levels demonstrated in these women of advanced reproductive age likely reflects the presence of a diminished follicular pool. The association between decreased inhibin B and elevated FSH concentrations suggests that inhibin B may be an important regulator of the monotropic FSH rise that occurs with aging, as well as being a biochemical index of prenatal follicles. This concept of inhibin B acting as a marker of reproductive age is supported by clinical data. These show that women with low day 3 serum inhibin B levels demonstrate a poorer response to ovulation induction and are less likely to conceive a clinical pregnancy through assisted reproductive technology than do women with high day 3 inhibin B levels [92]. By contrast, Klein

et al. [90] observed no difference in the day 3 inhibin A levels between older and younger women.

Seifer *et al.* [92] investigated whether inhibin B might be the physiological basis for the clomiphene citrate challenge test, commonly used to assess ovarian reserve. Clomiphene citrate blocks estradiol effects on the pituitary and induces increased FSH levels after treatment. The findings of this study demonstrated a decrease in inhibin B serum concentrations and a negative correlation with FSH levels in women with diminished ovarian reserve on day 3 and day 10. Taken together, these data suggest that inhibin B levels reflect the number of follicles present, whereas inhibin A may be a marker of the quality of a mature follicle.

Previous studies indicate that the menstrual cycles of older reproductive age women are characterized by a selective elevation of FSH associated with early development and ovulation of a dominant follicle. A study by Klein *et al.* [90] examined follicular fluid hormones and growth factors in the dominant follicle during unstimulated cycles of older ovulatory women. This was analyzed for estradiol, progesterone, testosterone, androstenedione, inhibin A and inhibin B, total activin A, total follistatin, IGF-I, IGF-II, IGF-binding protein-2 (IGFBP-2), IGFBP-3, and vascular endothelial growth factor (VEGF) concentrations. They demonstrated that the dominant follicles from older women contain normal concentrations of steroids, inhibin A and inhibin B, IGF-II, IGFBP-2, and IGFBP-3; increased concentrations of follistatin, activin A, and VEGF; and decreased concentrations of IGF-I. The elevated follicular fluid activin A may be related to the early ovulation observed in older women, whereas elevated VEGF may be related to the meiotic spindle abnormalities observed in the oocytes of older reproductive age women.

4. Pregnancy

During pregnancy, the placenta is the main source of inhibin and high serum levels of inhibin are reported [93–96]. There is a rapid decrease in inhibin concentration after delivery [97–101]. Specifically, total inhibin rises after ovulation, peaks at 9–10 weeks of gestation, reaches a plateau at $\sim$15 weeks, and then rises again in the third trimester.

Inhibin A is the principal (bioactive) form in pregnancy [96]. However, inhibin B levels are extremely low during early gestation, as neither the corpus luteum nor trophoblasts secrete this protein into circulation. Although inhibin β_B subunits have previously been detected in the placenta [102–104], their level of expression is considerably lower than that of either α or β_A [104, 105]. Although the β_B is heavily expressed in the amnion [102],

the virtual absence of inhibin B from circulation suggests that if the amnion synthesizes inhibin B, it should have a local action, possibly involving prostaglandin synthesis [102, 103]. The corpus luteum is known to make a significant contribution to the circulating inhibin concentrations during pregnancy [103, 106, 107, 108]. It has been demonstrated that treatment with exogenous human chorionic gonadotropin (hCG) produces a marked rise in the peripheral concentration of ir-inhibin [104].

Illingworth *et al.* [95] demonstrated that stimulation and maintenance of luteal function with hCG resulted in an increase in inhibin A concentration. In contrast to inhibin A, concentrations of inhibin B continued to fall in the late luteal phase despite maintenance of luteal function. This suggests that the corpus luteum is not a source of inhibin B and that the peak of inhibin B seen early in the normal luteal phase represents the passage of inhibin B from the follicular fluid into circulation rather than *de novo* synthesis [95]. The absence of detectable mRNA for the β_B subunit in either luteinized granulosa cells or the corpus luteum of women has been reported [64, 106].

It has been demonstrated that inhibin and activin influence hormonogenesis in cultured placental tissue, with activin increasing hCG and progesterone production. Activin measurements have been carried out by several groups using a bioassay [109] or an RIA [110] despite the difficulties of measuring activin due to its high affinity binding to follistatin. With the development of a total (bound + free) assay [111], Muttukrishna *et al.* [94] measured activin A throughout the pregnancy. By 8 weeks of gestation, serum activin A concentrations were higher than those during the normal menstrual cycle. The concentrations of activin A did not vary significantly during the remainder of the first and second trimesters. After 24 weeks, there was an increase in inhibin A and activin A near term, suggesting a role for these proteins in labor. Furthermore, studies confirm the presence of pro-αC, follistatin [112, 113], inhibin A, and activin A in circulation during pregnancy [114, 115].

4.1. Inhibin in Abnormal Pregnancy

There are identifiable differences in inhibin A production during normal pregnancies compared with abnormal pregnancies (Figs. 2 and 3). Seifer *et al.* [116] compared inhibin A and inhibin B levels in 19 women with confirmed ectopic pregnancy and in 24 women of similar gestational age with ultrasound-confirmed intrauterine pregnancy [116]. Both total and dimeric inhibin levels in the patients who had ectopic pregnancies were less than 60% of those in women with normal intrauterine pregnancy. We have noted this difference with respect to spontaneous abortions, as there was no difference in inhibin B levels in women undergoing a miscarriage compared with normal gestational controls [117].

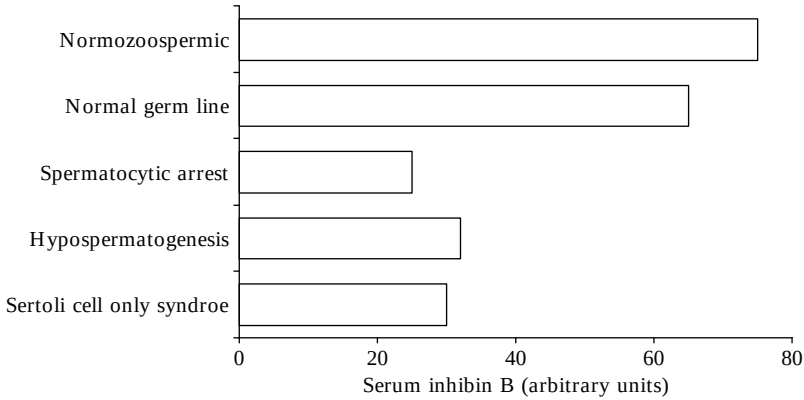

FIG. 2. Serum inhibin A concentration in gestational diseases.

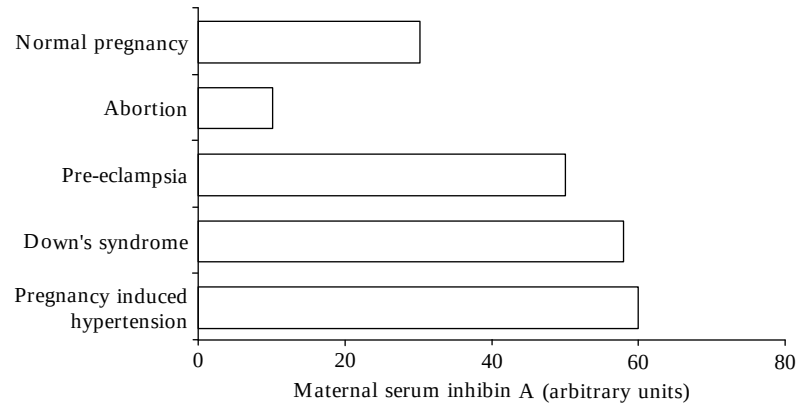

FIG. 3. Serum activin A concentration in gestational diseases.

4.2. PRE-ECLAMPSIA

Pre-eclampsia is one of the most serious conditions during pregnancy, as it markedly increases the risk of prenatal morbidity and mortality of both mother and fetus. Pre-eclampsia is defined as hypertension with proteinuria, edema, or both induced by pregnancy after 20 weeks of gestation [118].

Intense effort has been devoted to develop screening tests for pre-eclampsia and biochemical markers that could predict the subsequent development of disease [119–133]. Inhibin A and pro-αC subunit levels are increased in pre-eclampsia and show a positive correlation with hCG; thus inhibin A might be

a paracrine modulator or, at least, an additional marker of the placental overgrowth that characterizes this disease [114]. There is a significant increase in the levels of mRNA for inhibin α and β_A in the placenta of those with pre-eclampsia compared with controls, suggesting that the placenta is a source of the increased serum inhibin levels in pre-eclampsia [125, 134]. Serum activin A and inhibin A concentrations are tenfold higher in women with severe pre-eclampsia compared with gestational age-matched controls [126]. Furthermore, studies have demonstrated that activin A and inhibin A concentrations are elevated prior to the onset of pre-eclampsia [129]. It has been observed that inhibin A is increased at midtrimester [122, 123] with a twofold increase in the median among women who later developed pre-eclampsia [124], while inhibin A may be altered already in the first trimester (Table 2).

A combined measurement of maternal serum inhibin A and other uteroplacental markers may be an effective means of screening for pre-eclampsia in the second trimester of pregnancy [130]. The combination of the values of inhibin A, free β subunit of hCG, and uE3 to form a screening test would detect an estimated 55% of affected pregnancies, with a false positive rate of 5% [124]. There is no difference in serum inhibin B levels observed between healthy controls and patients at risk but who did not develop hypertension [117].

Hamasaki *et al.* [131] evaluated maternal serum ir-inhibin concentrations in women with pre-eclampsia, and assessed the correlation between serum ir-inhibin and hCG. The study found that there were no significant differences in maternal characteristics between the pre-eclamptic group and the

TABLE 2
POSSIBLE CLINICAL APPLICATIONS OF INHIBIN A AND ACTIVIN A MEASUREMENT DURING PREGNANCY IN MATERNAL SERUM

Phase of gestation	Predictive/diagnostic marker	References
First trimester		
Impending abortion	Inhibin A	Luisi *et al.* [162] Florio *et al.* [163]
Pre-eclampsia	Inhibin A	Zwahlen *et al.* [164]
Second trimester		
Down's syndrome	Inhibin A	Malone *et al.* [165]
Fetal demise	Activin A (amniotic fluid)	Petraglia *et al.* [166]
Pre-eclampsia	Inhibin A/activin A	Florio *et al.* [167]
Third trimester		
Pre-eclampsia	Inhibin A/activin A	Muttukrishna *et al.* [168]
Pregnancy-induced hypertension	Inhibin A/activin A	D'Antona *et al.* [169]
Intrauterine growth restriction	Inhibin A/activin A	Florio *et al.* [134]
Preterm delivery	Activin A	Farina *et al.* [170]

control group. The pre-eclamptic group had significantly higher concentrations of serum ir-inhibin and hCG compared with the control group. The serum concentrations of ir-inhibin correlated positively with those of hCG. Pre-eclamptic patients displayed high serum levels of ir-inhibin and hCG, and this might reflect hyperplasia of trophoblastic cells.

Also, urinary concentrations of activin A and inhibin A are altered in pre-eclampsia and the relationship between uterine vein and peripheral vein concentrations of these hormones in pre-eclamptic patients [132]. Urinary activin A and inhibin A are raised in groups 2 and 3 pre-eclamptic patients, thus suggesting that these proteins may rise in patients before the onset of the clinical symptoms of pre-eclampsia.

4.3. Down's Syndrome

Total or partial trisomy of chromosome 21 is responsible for the occurrence of Down's syndrome, which is the most common and severe abnormality at birth. Down's syndrome is characterized by an alteration in the secretion of placental and fetal proteins and steroids. Hence, the measurement of maternal serum markers has been shown to be useful in the screening for Down's syndrome [133, 135, 136]. Numerous pregnancy-associated maternal serum markers for Down's syndrome have been evaluated. Efforts to improve biochemical screening have centered on the search for a better marker in order to improve the detection rate or to reduce the number of false positives.

The levels of dimeric inhibin A are elevated in the maternal serum of women carrying a Down's syndrome pregnancy [135, 137], whereas inhibin B, pro-αC [138], and activin A levels [124] are not altered, at least between 16 and 19 weeks of gestation. These changes suggested a possible clinical role for measuring inhibin A levels in the biochemical screening of Down's syndrome.

Wenstrom *et al.* [139, 140] compared all combinations of α-fetoprotein (AFP), free β subunit of hCG, unconjugated estriol (uE3), and inhibin A. They concluded that the best triple test for screening was AFP, free β subunit of hCG, and inhibin A. In further studies, this group concluded that the multiple-marker test plus inhibin was superior to the traditional multiple-marker screening test and two other analyte combinations, with lower false-positive rates and increased detection of all aneuploidies in a high-risk population [140]. Subsequent data confirmed these observations [141].

In Down's syndrome pregnancies, median inhibin A levels were found to be significantly elevated in placental extracts and in maternal serum, when compared with the levels in uncomplicated pregnancies. Median activin A was also elevated in placental extracts and in maternal serum [142].

A dissociation between inhibin and activin subunit mRNA levels and the corresponding protein levels in maternal serum, and an increase in inhibin A levels that is not explained by mRNA upregulation has been reported in Down's syndrome [143]. In an addition to this study, ovarian cortex tissue from term pregnancies was examined. Only the α subunit mRNA was expressed at a higher level than in the placenta, suggesting that the ovary could be a source of inhibin pro-αC during pregnancies [143]. Table 2 lists possible clinical applications of the measurement of inhibin A, inhibin B, and activin A in pregnancy.

5. Inhibins in Men's Reproductive Function

The physiological role of inhibin in men became clearer since the availability of specific inhibin A and inhibin B ELISAs led to the demonstration that the predominant form in the male is inhibin B [144, 145]. There is general agreement that the major source of inhibin in the testis is the Sertoli cell, and in culture of rat Sertoli cells a linear relationship exists between the number of cells in culture and the inhibin levels secreted into the medium [146]. These cells respond to FSH by the increased production of inhibin. At high doses, FSH produces an excess of free α subunits [146]. Thus, in testicular damage, elevated levels of free α subunits can be detected in the circulation [145]. Further support for these concepts emerged from a prospective study of the effects of chemotherapy on the testis and its influence on serum FSH and inhibin B levels [147]. In the same study, it was shown that following spermatogenetic damage, inhibin B levels decreased as FSH levels increased. They also noted that an increase in pro-αC levels, which contributed to the maintenance of ir-inhibin levels measured by an assay, detects both inhibin and subunit products (Fig. 2).

There is a positive relationship between the levels of inhibin B in the circulation of rats and the number of Sertoli cells present in the testes in a variety of experimental states [148]. These results highlight the possibility that inhibin B levels in serum may provide an index of Sertoli cell function. Indeed, several earlier studies using a bioassay for inhibin strongly suggested that testicular damage in rats was accompanied by a change in Sertoli cell function reflected by a decrease in inhibin secretion [149].

There are limited data from *in vitro* approaches to indicate the importance of inhibin-related proteins in a paracrine action in the testis. Activin A has been shown to stimulate spermatogonial mitosis when these cells are co-cultured with immature rat Sertoli cells *in vitro* [150]. The role of activin A in the maintenance of an unusual form of mitochondria was found only in meiotic and postmeiotic germ cells in the testis and ovary [151], and these

mitochondria are termed "condensed" forms. When primary spermatocytes are placed in culture, the condensed forms are lost or change back to the orthodox forms found in spermatogonial and somatic cells [152]. The addition of activin A to the culture medium maintains the mitochondria in the "condensed" state, indicating that activins produced by the germ cells or by the Sertoli cells can exert a paracrine and autocrine function [153].

Inhibin B secretion in the adult is partly, but not completely, under gonadotrophin control. Administration of GnRH to men with hypogonadotrophic hypogonadism results in an increase in blood inhibin B levels from undetectable concentrations into the normal range in many cases [154–156] (Fig. 4). A further degree of complexity has been demonstrated by the studies of Bilezikjian *et al.* who showed that significant changes in these proteins occurred during GnRH and testosterone action on the pituitary [157]. For instance, GnRH stimulated follistatin mRNA and decreases β subunit mRNA levels, whereas testosterone decreases the levels of follistatin and β subunit mRNA (Figs. 3 and 4).

In another study, the relationship between testosterone and inhibin B was investigated [158]. In this study, administration of testosterone enanthate resulted in a fall in inhibin B concentration and azoospermia was achieved. It is possible that gonadotrophin secretion was more completely suppressed by those regimes that resulted in a clear fall in inhibin B. Hence, the presence or degree of fall in inhibin B does not predict whether azoospermia will be achieved, but it may indicate the degree of suppression of spermatogenesis or the step at which spermatogenesis is arrested [159].

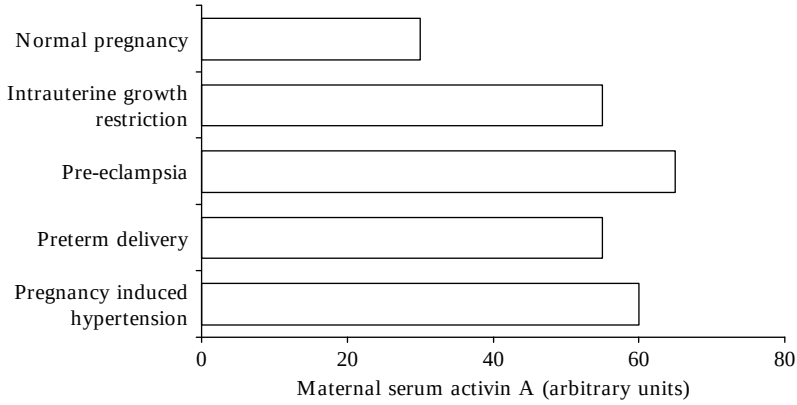

FIG. 4. Serum inhibin B concentration in man's reproductive function.

Aging in men is accompanied by a decline in reproductive function. A study has compared inhibin B concentrations in men aged 70–85 years with those in younger men [160]. The older men had lower inhibin B concentrations and high FSH concentrations, although the fall in inhibin was relatively modest (~25%) compared to the fourfold rise in FSH. The fall in inhibin B occurred at a relatively early age; thus the concentrations were lower in the 35- to 55-year-old age group compared with those younger than 35 years. The prevalence of low inhibin B concentrations was increased in the 35- to 55-year-old age group, whereas the prevalence of low testosterone concentrations appeared to rise only in the older groups [161]. The findings need to be confirmed, however, particularly the age at which inhibin B starts to fall. These studies indicate that inhibin B is the physiologically important form of inhibin in men. Although FSH stimulates inhibin B, there is also evidence for a gonadotrophin-independent component to its regulation in men. Inhibin B appears to play a significant role in the negative feedback loop regulating FSH secretion and may provide a useful marker of Sertoli cell function.

6. Conclusion

In humans, there appears to be important gender differences in the forms of inhibin present in the circulation. Both inhibin A and inhibin B are present in women, whereas only inhibin B circulates in physiological concentrations in men. In addition, there is evidence for a gonadotropin-independent component to inhibin B secretion in men; in fact, a dramatic switch from a positive to a negative relationship between inhibin B and FSH accompanies the initiation of puberty in boys [161]. The mechanism for this differential regulation in the male and female gonads is not known.

Measurement of dimeric inhibin A and inhibin B may provide sensitive diagnostic tools for determining gonadal maturity in early puberty of both genders, gonadal activity during reproductive life, and ovarian reserve in late reproductive years, besides monitoring feto-placental health at all phases of human gestation.

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uPA AND PAI-1 IN BREAST CANCER: REVIEW OF THEIR CLINICAL UTILITY AND CURRENT VALIDATION IN THE PROSPECTIVE NNBC-3 TRIAL

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1. Abstract

The plasminogen activator system is a complex system with multiple interactions and members participating in fibrinolysis, cell migration, angiogenesis, wound healing, embryogenesis, tumor cell dissemination, and metastasis in a variety of solid tumors. Increased levels of uPA and/or PAI-1 in primary tumor tissues of breast cancer patients correlate with tumor aggressiveness and poor clinical outcome. Patients with high tumor tissue antigen content of uPA and/or PAI-1 have a worse probability of disease-free and overall survival than patients with low levels of both of the biomarkers, serving as prognostic markers. The clinical utility of uPA and PAI-1 has

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been proven on the highest level of evidence (LOE-I). Next to being clinically useful prognostic factors allowing estimates of the course of disease in early breast cancer, uPA and PAI-1 may also serve as predictive factors predicting response to systemic therapy. Node-negative primary breast cancer patients with high uPA/PAI-1 levels benefit significantly from adjuvant chemotherapy. The aim of the ongoing NNBC-3 trial is to determine the benefits of a sequential anthracycline–docetaxel regimen in high-risk node-negative breast cancer patients compared to the current standard of anthracycline-based chemotherapy. At present, uPA and PAI-1 provide the unique opportunity to allow validated and clinically relevant risk assessment of breast cancer patients, over and above that provided by established risk factors. Therefore, in the evidence-based, annually updated AGO guidelines for breast cancer management, the German Working Group for Gynecological Oncology (AGO) has recommended both biomarkers as risk-group-classification markers for routine clinical decision making in node-negative breast cancer, next to established clinical and histomorphological factors.

2. The uPA/PAI-1 System

The plasminogen activator system, also known as the fibrinolytic system, consists of the serine protease-type plasminogen activators uPA (urokinase-type plasminogen activator) and tPA (tissue-type plasminogen activator), the high-affinity cell surface-associated receptor for uPA (u-PAR; CD87), the plasminogen activator inhibitors type 1 and 2 (PAI-1, PAI-2), and the proenzyme plasminogen which is activated by uPA or tPA into the proteolytically active serine protease plasmin. Although uPA and tPA are quite similar in structure and have common inhibitors and physiological substrates, their physiological roles are distinct [1]. This system is not only highly specific in catalyzing plasminogen into plasmin by tPA present in the blood stream (in the presence of fibrin) but is also effective in tissues when uPA is attached to its cellular receptor uPAR leading to activation of nearby plasminogen and subsequently to degradation of extracellular fibrin and other matrix proteins by the newly generated plasmin [2–5].

The plasminogen activator system is a complex system with multiple interactions among its members and also with constituents of the extracellular matrix. Traditionally, the role of tPA was primarily in fibrinolysis and that of uPA in cell migration, especially in angiogenesis, wound healing, embryogenesis, tumor cell dissemination, and metastasis [6–10]. The functional role of the plasminogen inhibitors is no longer simply to inhibit plasminogen activators: for PAI-1, a role in promoting cycles of attachment and detachment of the cell from the extracellular matrix that is independent

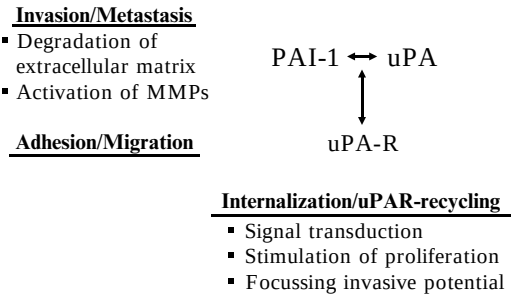

FIG. 1. Key role of the uPA/PAI-1 system in tumor invasion and metastasis.

of its role as an enzymatic inhibitor of uPA was revealed; PAI-2 has an unidentified role in the regulation of cell death, where it can alter gene expression, influence the rate of cell proliferation and differentiation, and inhibit programmed cell death (apoptosis) in a manner independent of urokinase inhibition [11,12].

Well-balanced production and activation of uPA/PAI-1 system components therefore can lead to changes in degradation of the extracellular matrix and also affect cell adhesion, angiogenesis, cell proliferation, and cell invasion, not only under physiological conditions but also under pathological conditions such as cancer (Fig. 1). Expression of uPA and PAI-1 is often higher in the epithelial and stromal cells of the tumor tissue than in the surrounding normal tissue pointing to the fact that in cancer, regulation of uPA-mediated extracellular proteolysis does involve a complex interplay between cancer cells, nonmalignant stroma cells, and components of the plasminogen activation system [13].

3. Clinical Relevance of uPA and PAI-1 in Breast Cancer

Elevated expression of uPA and PAI-1 at the mRNA and protein level was demonstrated in basically every solid malignant tumor type examined, such as cancer of the breast, ovary, esophagus, stomach, colorectum, kidney, lung, or liver. Increased levels of uPA and/or PAI-1 in primary tumor tissues correlate with tumor aggressiveness and poor patient outcome [9]. Among all of the articles published in the scientific literature on this subject, most data regarding the prognostic and predictive value of uPA/PAI-1 and their impact on clinical decision making are available for breast cancer. These data consistently show that high levels of uPA and/or PAI-1 determined in primary tumor tissue extracts by protein analysis or mRNA screening are associated with poor clinical outcome of the patient [14–22].

3.1. Prognostic Impact of uPA and PAI-1

In primary breast cancer, the validation process demonstrating the clinical utility of uPA and PAI-1 was achieved by several, mostly European, research centers. In 1988, Duffy *et al.* were the first to show that the serine protease uPA has a prognostic impact on breast cancer patients. They demonstrated that high enzymatic activity of uPA in primary breast cancer tissues correlates with tumor size, number of lymph nodes involved, and with shorter disease-free survival when compared to patients with low uPA activity [23].

Soon after this initial observation, Jänicke *et al.* demonstrated in 1989 that determination of the uPA antigen content in primary tumor tissue by enzyme-linked immunosorbent assay (ELISA) also allows prediction of the course of disease in patients who did not receive any adjuvant treatment [24, 25]. At the same time, it also became apparent that not only the antigen level of uPA but also that of PAI-1 possesses prognostic value in node-negative and node-positive breast cancer patients [26]. A model was then developed which still is of clinical relevance: Patients with high tumor tissue antigen content of uPA and/or PAI-1 have a worse probability of disease-free and overall survival than patients with low levels of both of the biomarkers [27–29]. Regarding risk-group assessment, the combination of uPA/PAI-1 (both low vs either/or both high) was found to be superior to either factor taken alone. Furthermore, based on multivariate analyses, uPA and PAI-1 are statistically independent of established prognostic factors such as tumor size, tumor grade, steroid hormone receptor status, or menopausal status [29]. As a prognostic factor, uPA/PAI-1 is superior to the oncoprotein HER2 and thus renders statistically independent, clinically important information [30, 31]. Other international research groups reported a similar prognostic impact of uPA and PAI-1 in breast cancer [31–34]. Moreover, the published interaction between PAI-1 and vascular endothelial growth factor (VEGF) warrants further investigation into the relationship between biomarkers of angiogenesis and those of the protease cascade [34, 35].

In order to achieve the highest level of evidence (LOE-I) for clinical utility of a cancer-associated biomarker, according to the ASCO tumor marker utility grading system (TMUGS) [36], clinical significance of a prognostic (or predictive) cancer biomarker must be evaluated either by a prospective randomized clinical trial or a large meta-analysis. Regarding this guideline, for uPA/PAI-1, both the criteria were fulfilled by: (1) a prospective randomized multicenter breast cancer therapy trial (Chemo N0) in which a total of 689 patients were enrolled in 14 study centers in Germany and Slovenia between 1993 and 1998 [37]; (2) a large meta-analysis conducted by the EORTC Receptor and Biomarker Group, comprising 8377 breast cancer patients from 18 independent datasets [38] (EORTC = European Organisation for Research and Treatment of Cancer; a trans-European cancer foundation based in Brussels, Belgium).

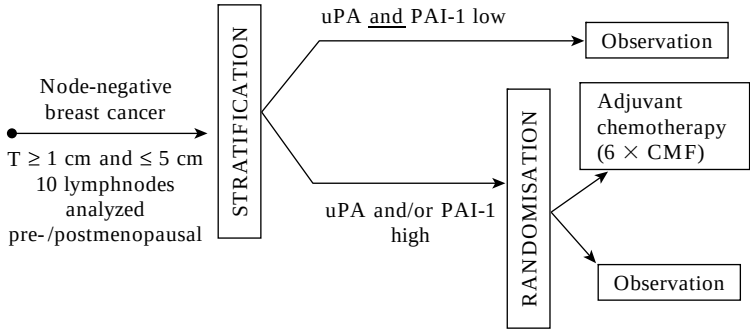

FIG. 2. Study design of the Chemo N0 trial [17].

In the Chemo N0 trial (Fig. 2), uPA and PAI-1 antigen content were determined in primary tumor tissue extracts by commercially available ELISA kits obtained from American Diagnostica Inc., Stamford, CT, USA. Patients with low uPA and PAI-1 content were allocated to the observation arm; patients with high uPA and/or PAI-1 content were randomized to either adjuvant chemotherapy with six cycles of CMF (cyclophosphamide/methotrexate/5-fluorouracil) or observation only. By comparing the two observation arms (low vs high uPA/PAI-1), the prognostic impact of uPA/PAI-1 could be evaluated in a prospective fashion. The first interim analysis of the Chemo N0 trial after a median follow-up time of 32 months demonstrated a statistically independent prognostic impact of uPA/PAI-1 with regard to disease-free survival. Also, previously optimized cut-off values for uPA and PAI-1 to discriminate between low and high uPA/PAI-1 were confirmed [37]. A second analysis after a median follow-up time of 50 months comprising 647 patients consolidated the prognostic impact of uPA/PAI-1 regarding disease-free and overall survival. This analysis revealed that node-negative breast cancer patients presenting with low uPA/PAI-1 in their primary tumor tissues have a low-risk profile with a 5-year overall survival rate of ~95% even in the absence of any adjuvant systemic therapy [39].

3.2. PREDICTIVE IMPACT OF uPA AND PAI-1

Next to being clinically useful prognostic factors allowing estimates of the course of disease in early breast cancer, uPA and PAI-1 may also serve as predictive factors predicting response to systemic therapy. As yet, only few studies have been published looking at the predictive impact of uPA and

PAI-1 regarding response or failure to chemotherapy or endocrine therapy in breast cancer. For instance, in the palliative setting, it was shown that metastasized breast cancer patients with high levels of uPA and PAI-1 in their primary tumor tissue responded poorly to palliative endocrine tamoxifen therapy, compared to patients with low levels of both factors [40, 41]. In the neoadjuvant setting, Pierga *et al.* looked at the correlation between PAI-1 levels determined in drill biopsy tumor tissue samples taken before anthracycline-containing neoadjuvant chemotherapy and in tumor biopsies taken after chemotherapy at the time of primary surgery in a small collective of 69 patients. No association was found between initial PAI-1 levels and clinical response to primary chemotherapy [42].

In early breast cancer, the improvement in clinical risk assessment and therapy benefit prediction gained by combining uPA and PAI-1 was evaluated by Harbeck *et al.* [29]. For this, uPA and PAI-1 levels were prospectively measured by ELISA in tumor tissue extracts of 761 patients with primary breast cancer. The criterion either alone or by both factors has identified with high sensitivity the patients at high risk for disease recurrence while keeping more than half of the patients in the low-risk group. More interestingly, a significant interaction between uPA/PAI-1 and adjuvant systemic therapy was demonstrated suggesting a benefit from adjuvant therapy in high-risk breast cancer patients as defined by uPA/PAI-1. Harbeck *et al.*, in a subsequent study of 3424 primary breast cancer patients from breast cancer centers in Munich, Germany, and Rotterdam, The Netherlands, evaluated the predictive impact of uPA/PAI-1 regarding response to adjuvant chemo- and endocrine therapy [43]. It was shown that patients with high uPA/PAI-1 levels had an enhanced benefit from adjuvant chemotherapy compared to those with low levels of the biomarkers. No corresponding interaction between endocrine therapy and low or high uPA/PAI-1 levels was observed. The uPA/PAI-1-dependent benefit from adjuvant chemotherapy was subsequently validated in the pooled analysis collective of the EORTC Receptor and Biomarker Group [44].

In the prospective multicenter Chemo N0 trial, node-negative primary breast cancer patients with high uPA/PAI-1 levels were either randomized to CMF-based chemotherapy or to observation only, thereby looking at the clinical value of a therapeutic intervention in high uPA/PAI-1 patients. Already after a short follow-up period of 32 months, a considerable and statistically significant benefit from adjuvant CMF chemotherapy was also observed in high uPA/PAI-1 node-negative breast cancer patients, which was still valid after a longer median follow-up time of 50 months [37, 39]. This prospective randomized multicenter clinical trial therefore not only validated the prognostic value of uPA/PAI-1 in node-negative breast cancer patients but also demonstrated their predictive impact at the highest level of evidence, LOE-1.

4. Methods for Determination of uPA and PAI-1

In the majority of published retrospective studies, quantification of uPA and PAI-1 antigen levels in tumor tissue extracts was performed using the commercially available ELISA kits [27]. These assays were also used for the prospective Chemo N0 clinical trial where routine tissue analysis for clinical decision making within the trial was conducted in six different laboratories [37]. Consequently, for routine use, quantitative determination of uPA and PAI-1 antigen content by ELISA using these assays is recommended, employing protein extracts from fresh-frozen primary breast cancer tissue samples. These assays can also be applied to assess protein extracts obtained from small breast cancer biopsies, such as core biopsy specimens or cryostat sections [45]. The uPA and PAI-1 ELISA tests have been standardized and quality assured by the Receptor and Biomarker Group and the PathoBiology Group of the EORTC [46–48].

At present, PCR-based mRNA analysis of uPA and/or PAI-1 messenger expression has not reached the level of clinical routine, mainly due to the lack of validated, multicenter clinical studies. Still, such studies should be encouraged as recent publications, although on a few patient collectives only, indicated feasibility of such a test system for breast cancer tissue uPA/PAI-1 analysis, which would be independent of protein expression or internalization of uPA/PAI-1 complexes by the cells [49–52].

Likewise, no validated multicenter clinically relevant breast cancer immunohistochemistry studies, using uPA and/or PAI-1 directed antibodies and routinely processed paraffin-embedded formalin-fixed breast cancer specimens, have been conducted so far.

Consequently, harmonization of various detection and quantification systems for uPA and PAI-1 at the gene and protein level is one of the current tasks of the EORTC PathoBiology Group. In this context, we would like to mention that in addition to mRNA and protein expression, epigenetic modification of CpG islands within the promoter region of the uPA gene is also an indicator of tumor aggressiveness; still, prospective breast cancer trials observing uPA DNA-methylation as a stratification factor have not yet been conducted [53, 54].

5. Clinical NNBC-3 Trial

As a consequence of the results of the Chemo N0 breast cancer trial, a number of clinical questions were answered but others still remain open regarding prognosis and therapy response prediction of breast cancer patients.

Breast cancer patients with low levels of uPA and PAI-1 comprising about half of the node-negative breast cancer patients have a rather low risk of disease recurrence (<10%) and therefore are not subjected to adjuvant systemic chemotherapy. In contrast, patients with high uPA and/or PAI-1 levels, who are at risk to develop metastasis do benefit from adjuvant systemic therapy.

A second prospective multicenter phase III therapy trial, the NNBC-3 trial (Node Negative Breast Cancer 3-trial) is now open for patient recruitment [Principal investigators: Prof. Dr. Christoph Thomssen ("Leiter der Klinischen Prüfung" according to German law), University of Halle; and Prof. Dr. Nadia Harbeck, Technical University of Munich, Germany]. The two main aims of this trial are: (1) to compare risk assessment and clinical outcome based on tumor biological factors uPA/PAI-1 to that based on established, clinical and histomorphological factors; and (2) to optimize the adjuvant chemotherapy regimen for high-risk node-negative breast cancer patients. The NNBC-3 trial is an intergroup trial conducted in cooperation with the AGO (German "Arbeitsgemeinschaft Gynäkologische Onkologie"), the EORTC PathoBiology Group, and the German Breast Group. By end of October 2007, recruitment was at 2022 patients who came from 97 clinical centers in Germany and France. Node-negative primary breast cancer patients aged 18–70 years with a tumor size between 0.5 and 5 cm are eligible for the trial. Recruitment centers are allowed to select risk assessment criteria either using established clinical and histomorphological criteria or using determination of uPA/PAI-1 in primary tumor tissue extracts. Risk assessment by established factors takes the recommendations of the St. Gallen 2005 consensus meeting into account (Fig. 3A) [55]: Using these criteria, a node-negative breast cancer patient fulfilling any of the following criteria is regarded as being at high risk for disease recurrence: (1) age <35 years, peritumoral vascular invasion, tumor grade 3, steroid hormone receptors PgR−/ER+ or PgR−/ER−, or HER2-positive, or (2) tumor grade G2 and tumor size >2 cm.

The second option for risk assessment is based on the presence of the tumor biological factors uPA and PAI-1 in the primary tumor tissue. However, for risk assessment, node-negative primary breast cancer patients are first classified according to tumor grade. Patients with G1 tumors are considered at low risk and allocated to the observation arm; all G3 tumor patients are considered at high risk and therefore are randomized in the chemotherapy arm. For patients with G2 tumors, further risk stratification according to uPA/PAI-1 is appropriate. Patients with 35 years of age or above and low uPA/PAI-1 are considered at low risk and thus allocated to the observation arm. Patients with 35 years of age or above and high uPA and/or PAI-1, or those younger than 35 years of age, are randomized to one of the two chemotherapy regimens.

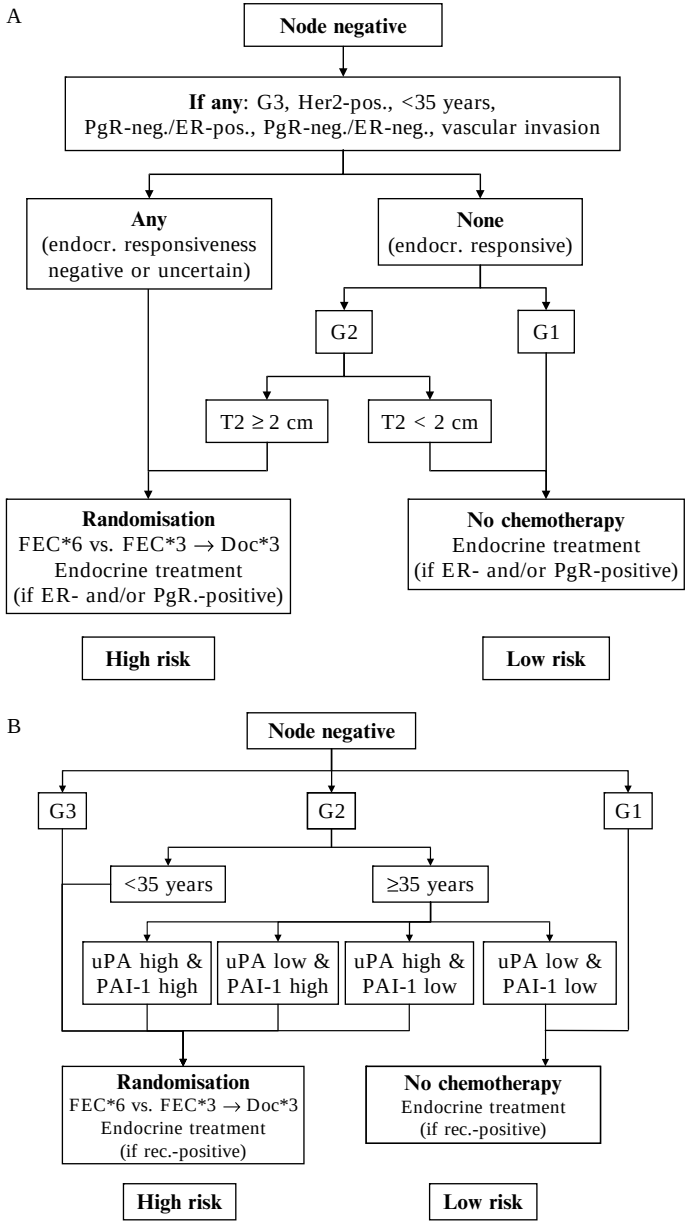

FIG. 3. (A) NNBC-3 trial: clinical and histomorphological risk assessment. (B) NNBC-3 trial: tumor biological risk assessment.

In the NNBC-3 trial, all patients defined as being at high risk for disease recurrence are randomized to either six cycles of $FE_{100}C$ (5-fluorouracil/epirubicin/cyclophosphamide) every three weeks or to three cycles of $FE_{100}C$ followed by three cycles of docetaxel every three weeks. Node-negative patients defined as being at low risk for disease recurrence are allocated to the observation arm and receive endocrine therapy according to the AGO guidelines if applicable [56].

The overall aim of the trial is to compare both risk stratification methods—the clinical and histomorphological and the tumor biological one—and to determine the benefit of a sequential anthracycline–docetaxel regimen in high-risk node-negative breast cancer patients.

We would like to mention that another clinical therapy trial, the ADEBAR trial (Adjuvant Docetaxel versus Epirubicin-Based Regimen Trial), has already been completed in which ~1500 high-risk node-positive breast cancer patients with more than three involved axillary lymph nodes were enrolled. Patients recruited in 198 centers were randomized to either six cycles of $FE_{120}C$ or to six cycles of a three-weekly sequential anthracycline–docetaxel regimen. Testing for uPA and PAI-1 was an optional translational research subprotocol intended to evaluate the benefit of adding taxane to anthracycline-based chemotherapy in the two risk groups according to the uPA/PAI-1 status. The ADEBAR trial was closed for patient recruitment in spring 2005; clinical results and thus also results of the uPA/PAI-1 subprotocol are still pending.

6. Current Use of uPA/PAI-1 in Clinical Decision Making

Tumor biological factors uPA and PAI-1 were validated at the highest level of evidence regarding their prognostic and predictive impact in primary breast cancer. Therefore, the German Working Group for Gynecological Oncology (AGO) recommends both biomarkers for risk-group-classification and routine clinical decision making in node-negative breast cancer next to the established clinical and histomorphological factors. These recommendations were first issued in 2002 in the annually updated evidence-based AGO guidelines (www.ago-online.org) (Fig. 4) [56].

Still, although substantial and consistent data have been published and prospective clinical therapy trial evidence validating these prognostic factors and demonstrating the benefit of adjuvant chemotherapy in high uPA and/or PAI-1 node-negative breast cancer patients is available, these tumor biological markers have until recently not been integrated in international guidelines, such as the St. Gallen conference consensus [55]. While the clinical validity is not being disputed, one reason for the limited worldwide

A

Prognostic factors
in node negative breast cancer

Factor	Oxford LOE/GR		AGO
Tumor grade	2b	B	++
Tumor size	2b	B	+
Age	2b	B	+
uPA/PAI-1	1a	A	+
Proliferation (SPF, TLI, Ki67/MiB1)	2b	B	+/–

AGO - Leitlinie 2006: www.ago-online.org

B

Predictive factors
adjuvant therapy

Treatment	Factor	Oxford LOE/GR		AGO
Endocrine therapy	ER/PR status	1a	A	++
	Her-2	2b	D	–
Ovarian ablation	Menopausal status	1c	A	++
Aromatase inhibitors	Menopausal status	1c	A	++
Chemotherapy	HER-2	2b	D	–
	uPA/PAI	2a	C	+/–
Trastuzumab	HER-2	2b	D	++

AGO - Leitlinie 2006: www.ago-online.org

FIG. 4. AGO 2006 Breast Cancer Treatment Guidelines: prognostic (A) and predictive (B) factors for clinical routine use.

acceptance is certainly the necessity of fresh-frozen tumor tissue for preparation of tumor tissue extracts and ELISA testing, which is not available as a standard of care in many countries of the world, including USA. This is rather surprising since for many years fresh-frozen breast cancer tissue was set aside for steroid hormone receptor testing by the DCC or EIA test. Moreover, mRNA testing employing cDNA-microarray technology also requires fresh breast cancer tissue for analysis [57, 58]. Based on the level I evidence, the most recent ASCO guidelines 2007 have included uPA and PAI-1 as markers which are recommended for use in practice.

We want to stress again, that to date uPA and PAI-1 are the only breast cancer-associated biomarkers whose clinical utility was demonstrated by a prospective clinical trial (Chemo N0) with a second confirmatory trial (NNBC-3) on its way.

Regarding prospective gene signature validation in breast cancer patients, the first clinical therapy trials have just been launched, such as TAILORx (Trial Assigning IndividuaLized Options for Treatment, Rx), using the 16-gene disease recurrence score [59], or MINDACT (Microarray In Node-negative Disease may Avoid Chemo Therapy) using a 70-gene signature for risk assessment [57]. Thus, at present, only uPA and PAI-1 provide the unique opportunity to allow validated and clinically relevant risk assessment in breast cancer patients, over and above that provided by the traditional and established risk factors.

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ADVANCES IN MULTIPLE ANALYTE PROFILING

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1. Abstract

The advent of multiparameter technology has been driven by the need to understand the complexity in biological systems. It has spawned two main branches, one in the arena of high-content measurements, primarily in microscopy and flow cytometry where it has become commonplace to analyze multiple fluorescence signatures arising from multiple excitation sources and multiple emission wavelengths. Microscopy is augmented by topographical content that identifies the source location of the signature.

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The other branch involves multiplex technology. Here, the intent is to measure multiple analytes simultaneously. A key feature of multiplexing is an address system for the individual analytes. In planar arrays the address system is spatial, in which affinity reactions occur at defined locations. In suspension arrays, the address is encoded as a fluorescent signature in the particle assigned to a specific reaction or analyte. Several hybrid systems have also been developed for multiplexing.

In the commercial regime, the most widespread applications of multiplexing are currently in the areas of genome and biomarker analysis. Planar chips with fixed arrays are now available to probe the entire genome at the level of message expression and large segments of the genome at the level of single nucleotide polymorphism (SNP). In contrast, suspension arrays provide the potential for probing segments of the genome in a customized way, using capture tags that locate specific oligonucleotide sequences to specific array elements.

2. Introduction

High-content analytical techniques are rapidly being introduced into both research and clinical laboratories in response to a shift in perspective from the analysis of individual molecules to the analysis of complex biological systems. The task of undertaking large-scale analysis of biological interactions has been driven in part by recent advances in combinatorial chemistry, genomics, and proteomics. The proliferation of these approaches has been made possible by the expanding availability of tools that include reagent collections, shared databases, and analytical algorithms as well as miniaturization, automation, and cost-effective strategies that together are producing platforms for genome-scale analysis.

The power of multiple analyte profiling is in the utilization of technical and informational synergies in parallel systems, often in real time, revealing information among related analytes that may not be fully appreciated in conventional iterative assays. Simultaneous measurement of a number of different characteristics, a process that is referred to as multiparametric analysis, is beginning to be exploited in a number of applications. In multiplexing approaches, multiple sets of data are collected in a single reaction volume where each data set reports a unique analyte. For example, dozens and potentially hundreds of analytes can be measured simultaneously in microsphere-based systems [1]. Multiple bead sets are uniquely color coded with spectrally distinct fluorophores or with multiple levels of a single fluorophore, each engineered to monitor a reaction with a unique analyte. Beyond the rapid accumulation of data, these systems offer the ability to integrate

qualitative findings with robust analytical techniques for quantifying complex interactions among molecules.

This chapter reviews several platforms and applications of multiple analyte profiling which have matured substantially over the past decade, largely due to the impact that high-content platforms have had in genomics research. The utility of flow cytometry has been appreciated in nearly every area of experimental biology for over 30 years. A newly emerging class of flow cytometry-based applications is proving to be a highly effective tool set for the biophysical research laboratory. The breadth of performance, particularly in the area of multiprotein assembly, and the amenability of flow cytometry to multiplexing strategies is a focus of this chapter.

3. Multiple Analyte Profiling Platforms

Fluorescence-based cell, particle, and planar array assays are now routine practice in a variety of diagnostic screening applications including biomarker assays, genotyping, and immunologic profiling. In the context of bioanalytical platforms, information content refers to data capture capacity. It is driven by throughput (the analytical rate) and the biological complexity of the data (the diversity of the reagents and targets). High content specifically refers to making multiple measurements on, for example, a single cell population, whereas multiplexing would allow simultaneous measurements on multiple cell populations. The analysis of surface markers on blood leukocytes for vaccine development is an example of a high-content, multiplexed measurement [2]. Increasingly, multiplex formats are being advanced to increase information content for existing applications and for developing new approaches for conducting mechanistic studies of complex physiological processes.

3.1. Planar Arrays and Hybrid Bead-Based Systems

Spatially addressable fluorescence-based planar arrays for high-content assays revolutionized the field of genomics research. Within a decade of implementation, nucleic acid microarrays became the technological cornerstone for large-scale surveys of genes, transcripts, expression libraries, and SNPs. DNA microarrays are now providing excellent tools for exploring the gene expression patterns of tens of thousands of genes simultaneously.

The use of planar DNA microarrays represents a hallmark technology of multiplex analysis that has matured by building on the fundamental concept of simultaneous detection of multiple analytes to reduce time, labor, and cost as compared with single reaction-based methods. Implementation of early

DNA biochip array technologies soon became restricted by a rigid fabrication protocol, large feature sizes, and high-detection limits requiring large amounts of sample [3]. The introduction of techniques for *in situ* transcriptome amplification [4, 5] and cell-free *in situ* expression from PCR products [6], and the development of microfluidics handling and droplet microspotting [7, 8] have expanded the applications in systems with limited abundance of analyte. Further, advances in on-chip micromachining, and the availability of a growing variety of substrate chemistries have increased the flexibility of planar array constructions and their integration with other analytical systems.

Protein arrays for immunoassay applications soon became to the field of proteomics what DNA microarrays were to genomics. Clinical applications using planar arrays for human and veterinary disease surveillance, diagnostics, prognostics, and therapeutics management are now commonplace. Immunoassay techniques exploit the wide diversity and biological specificity of antigen binding by immunoglobulins, and affinity ligands include monoclonal antibodies or their ligand-specific domains. Microarray immunoassays using spotting techniques require picoliter amounts of analyte, and reaction volumes in the microliter range typically confer good signal-to-noise ratios and short diffusion distances that result in reduced reaction times [9].

The immunoassay is the conventional "gold standard" for protein measurement, with the enzyme-linked immunosorbent assay (ELISA) format reliably demonstrating detection limits of proteins in solution in the picogram per milliliter range over a dynamic range of 3 logs of concentration. There are many options for labeling and signal enhancement that are founded on the detection of ligand binding through the association of the ligand with a fluorescent, colorimetric, histochemical, or radioactive readout. Other formats that are based on immunoassay principles include flow cytometry and immunohistochemical and fluorescence microscopy.

Multiplex cytokine analysis provides a prime example of how multianalyte profiling confers context to the analysis of proteins beyond the measurement of abundance to include interaction, and modification by other antagonistic and synergistic proteins that is not achievable by single protein measurements. Protein planar microarrays are a validated platform for profiling analysis on a scale that far exceeds conventional ELISA methods [10–12].

The rapid adoption of flow cytometry-based assays for conducting multiplexed cytokine studies deserves some consideration. Current bead array technologies can easily accommodate 100 analytes simultaneously. A study comparing solid phase and bead-based cytokine assays using the same antibody pairs in each assay demonstrated improved detection limits using a bead-based multiplexed assay compared with ELISA analysis. The dynamic range of the bead-based assay in this comparative study also improved by 1 log [13].

Some recent examples of multiplexing strategies that have gone beyond descriptive science and into functional strategies for clinical disease management include vaccine development [14], isolation, and characterization of nucleic acid aptamers for research tools and therapeutics [15], the identification of tumor autoantigens and the advent of immunotheranostics [16], pharmacogenetic profiling for point-of-care diagnostics [17], and new parallel systems approaches for investigating, monitoring, and treating multifactorial polygenic diseases such as rheumatoid arthritis [18]. The introduction of cell-based microarrays has transformed cytogenetics from an *in vitro*-based platform for descriptive assays into an avenue for the development of *in vivo*-based analytical tools for mechanistic molecular biology [19].

Planar protein arrays have been used in high-throughput mechanistic studies of kinome profiling [20–22], systematic searches for antibody specificities [23], comprehensive studies of protein phosphorylation [24], carbohydrate recognition studies (functional glycomics) [25], DNA–protein binding interactions [26], RNA interference assays [27, 28], and ligand binding assays for G-protein–coupled receptors [29]. Microarrays using mixed protein suspensions or whole lysates from stimulated cells representing a full complement of intracellular proteins have recently been used to construct a comprehensive phosphorylation profile of CD3- and CD28-mediated signaling components [30].

A novel extension of the planar array concept for highly parallel genomic assays using fluorescence-based fiber-optic technology has been developed. Bundled, light-conducting fiber-optic strands (6000–50,000 per bundle), each chemically etched with a 3-μm well at the terminus, are configured into standard 96-, 384- or 1536-well spacing formats. The microwell array is capable of accommodating microspheres, each containing up to thousands of copies of a unique probe. The interrogation of extremely small sample volumes within the 3-μm wells has pushed the detection limit in this system into the zeptomolar range, or as few as 600 target DNA molecules [31]. The multiplexed assay detects up to 1536 SNPs from a single DNA sample. In genotyping assays, up to 300,000 data points can be generated per day and robotics platforms are capable of increasing throughput to over 1.5 million genotypes per day [32, 33].

The principles of microfluidics platforms for flow-through bead-based analytical systems have been reviewed by Buranda *et al.* [34] where multiplex analyses have been created in microcolumns with individual beads detecting individual analytes. Microchip-based immunoassay systems utilize porous microbead arrays positioned on silicon wafer platforms within microcavities, each serving as a miniaturized reaction vessel and analysis chamber. The utilization of porous beads allows for the application of a thick layer of capture reagents. A liquid chromatography system introduces solutions via

pressure-driven flow or capillary forces at a rate of 2 ml/min through a flow cell enclosing the silicon platforms. Colorimetric and fluorescent optical signals are acquired in near real-time digital analysis. A microchip-based system for point-of-care cardiac risk assessment has been described [35, 36] in which multianalyte analysis is conducted in 30 nl volumes with assay characteristics that are comparable and in some cases superior to macroscopic analytical platforms. In the cardiac risk assessment system, the detection limit for C-reactive protein, a major participant in the acute phase response, was 1 ng/ml compared with limits reported for other automated assay systems ranging from 5 to 80 ng/ml. The quantification and identification of electrolytes, sugars, proteins, antibodies, and toxins has also been demonstrated in this format.

High-throughput systems for label-free interrogation of microarrayed proteins by surface plasmon resonance imaging are capable of real-time biomolecular imaging of protein–DNA interactions on planar arrays with 1 s time resolution and subpicogram sensitivity [37, 38]. Aside from obviating the need for labeling, the advantages over competing techniques include low reagent requirements, minimal expense of mass-producing sensor chips compared with glass components of other systems, and simple requirements for grating-based sensing optics [39]. Arrays for real-time analysis of clinically relevant protein–protein (HPV E6, E6AP, and p53) and peptide–antibody interactions in complex biological fluids (anti-HCV antibodies in patient-derived sera) have recently been reported using surface plasmon resonance imaging [40, 41]. The technique has shown efficacy for detecting conformational properties of target proteins in a study using anti-Bax antibody chips to detect conformation-specific epitope alterations in cancer cells treated with an apoptosis-inducing ligand [42]. High-throughput screening applications of surface plasmon resonance have also been demonstrated in identifying small molecule inhibitors targeting protein–protein interactions [43], and in identifying new protein–protein interactions derived from a proteome-wide protein expression library [44].

3.2. High-Content Cellular Imaging

Imaging of complex physiological cellular processes has been adapted to high-content, high-throughput platforms. Detection systems include microscopy, fluorescence macroconfocal detectors, and fluorometric imaging plate readers. A recent review of automated cellular-imaging platforms [45] reveals that modern automated fluorescence and laser scanning platforms are capable of generating over 50,000 data points per day, and automated confocal systems >1 million data points per day. Throughput capacity combined with multiparametric biological measurements has expanded the use of

image-based platforms into functional analysis of biological systems. For example, high-content multiplex image analysis has been developed for simultaneous profiling of cell signaling pathways of up to 13 signaling molecules per CD33-positive myeloid cell isolated from cancer patient populations [46]. Phosphoprotein signaling profiles in the same study were used in both disease classification and target leads. In another multiplexed fluorescence microscopy application, detection and quantification of nuclear factor-κB (NF-κB), p38 and c-JUN translocation in response to inflammatory stimuli was used to distinguish compound selectivity among these pathways [47].

Resolution options allow for multicontent screens for imaging cell populations (e.g., phenotypic data) and subcellular events (e.g., protein translocations) in the same sample. In principle, sets of cell images obtained under a variety of optical environments (various fluorescence filter arrangements, brightfield/darkfield imaging) would provide comprehensive information about cell morphology and spatial distribution of any number of optical signals that is unachievable with any other single technology. Improvements in the spectral resolution of fluorochrome tags for simultaneous tracking of more events and better analytical algorithms for tracking events in multidimensional spaces will continue to move this technology to the forefront of high-content analytical methods.

3.3. Suspension Arrays/Flow Cytometry

A growing number of assays have been adapted to flow cytometry bead-based platforms with the capacity to function as multiplexed systems. In addition to the improved analytical performance for many assays, bead-based assays offer rapid analysis, high throughput, and robust quality control parameters [48]. Bead-based systems can accommodate a high diversity and a growing density of array features. For example, beads have been coated with authentic biological materials, such as lipid bilayers, to construct surface display libraries for the study of virus-specific antibodies [49], plasma proteins [50, 51], and interaction of cholera toxin with cell surface receptors [52]. A summary of the features of bead-based arrays and planar arrays is presented in Table 1.

As discussed previously, bead-based multiplexed platforms for serology profiling have the potential to replace many ELISA-based assays. A recent study of antibodies against recombinant HPV proteins reported highly reproducible ($R^2 = 0.97$) bead-based assay results with a dynamic range of 1.5 orders of magnitude and antibody detection at serum dilutions >1:1,000,000 and a CV of <5.4%. In this example and others, concordance with conventional ELISA approaches was high [59–61].

TABLE 1
COMPARISON OF PLANAR AND BEAD-BASED ARRAY FORMATS

	Planar	Bead-based
Probe density (no. of analytes)	Tens of thousands	Maximum of 100
Throughput (samples/day)	Hundreds [53]	Thousands [52]
Processing	Image processing	Standard flow cytometry
Automation	Under development [54]	Available [55]
Flexibility	Fixed array (new parameter requires new production)	Flexible (new parameter, add a new bead)
Quality control	Complex validation and analysis [56, 57]	Statistically robust [58]
Sensitivity with impure or complex samples	Superior to ELISA [54]	Comparable to ELISA [54]
Production/Instrumentation	Specialized, single use facility	Standard, multiuse flow cytometry

Microspheres have been readily adapted to flow cytometry-based multiplexing platforms due to their inherent stability, uniformity, and capacity for fluorescent dye uptake and retention. Surface chemistries are available for covalent coupling (via carboxyl, amine/hydrazide, and maleimide groups), noncovalent linkages, species-specific anti-IgG, and high/low-density streptavidin-coated fluorophores for assay optimization. Other affinity tags (glutathione-GST, Ni^{2+}-6x-histidine, and protein A and G) are available for linking capture proteins to microspheres. The breadth of bead technology continues to expand as new optically addressable particle-encoding approaches are explored.

Bead sets are commercially available for the simultaneous interrogation of up to 100 unique analytes. Planar array formats provide higher probe density; however, the throughput potential of planar arrays may not yet be fully realized because of limitations in processing and automation (see Table 1). In principle, implementation of any highly multiplexed immunoassay is limited only by inherent characteristics of assay chemistries that result in loss of quantitative response. For example, bead-based multiplex platforms are likely to face the same challenges as solid phase arrays with regard to the use of multivalent and cross-reactive immunological reagents, optimization across multiple assay conditions, and technical aspects of detection parameters to preserve overall sensitivity and specificity. Perlee *et al.* and others [56, 23] have recently reviewed these challenges and proposed standard methods for the validation and integration of data from different measurement technologies, including those for planar multiplexed antibody

protein arrays. Among the issues they have identified are antibody cross-reactivity under highly multiplexed conditions, robust statistical approaches to data management, quality control testing of reagents, and real-time monitoring of assay quality indicators (sensitivity, dynamic range, and platform precision). Other specific technical considerations for building bead-based assays are discussed in Section 5.4.

Jacobson *et al.* [62] used an assay model of microspheres covalently coupled with biotinylated BSA bound with titrated amounts of streptavidin, R-phycoerythrin (PE-SA) to demonstrate how non-bead-associated fluorescence determinations and instrument properties significantly affect bead array statistical sensitivity analysis and detection limits of the assay. The report details the effective use of instrument settings and statistical methods by using population parameters from each bead set for optimizing the quantification of ligand in array systems.

Iannone *et al.* [63] explored the effects of bead substrate binding site density on the K_{app} for soluble binding ligands using a nuclear receptor binding domain (PPARγ LBD) with an interacting synthetic peptide (PGC-1α) coupled to microsphere populations, each with a unique density of bound peptide. The study demonstrated that low-density ($<$200,000 molecules per microsphere) affinity for receptor–peptide interaction decreased proportional to peptide density, while high-density ($>$200,000) affinity did not vary. The matrix effects of avidity arising from high-local concentrations of neighboring immobilized peptides in close proximity increased the probability that the receptor will rebind another peptide rather than becoming free in solution (by dissociation). These effects can significantly reduce the apparent off-rate, resulting in higher-apparent affinities. Avidity effects can be exploited by intentionally coupling to high density and increasing assay sensitivity, but the increased avidity effect could reduce potential selectivity of assay. The study findings have important practical implications for optimizing conditions for multiplexed analytes with differing affinity characteristics.

Flow cytometry enables the simultaneous quantitative analysis in individual cells or particles of multiple optical markers of biochemical expression or physiological response. Microsphere-based studies of complex molecular interactions have been appreciated for nearly a decade [64]. Even the most inexpensive modern instruments can measure five optical parameters at once (three fluorescence and two light scatter signals). Flow cytometry is thus an inherently high-content quantitative methodology. It is also a sensitive technology, capable of detecting fluorescent molecule concentrations as low as 10–100 pM, and as few as hundreds to thousands of molecules on a cell or bead. Moreover, due to the optical configuration, the laser in a flow cytometer excites only a very small volume of the sample fluid immediately

surrounding the cell or the bead [55]. This allows discrimination of free and particle-bound fluorescent probe over a large range of probe concentration up to at least several hundred nanomolar. Consequently, homogeneous (no wash) assays may be implemented to streamline sample processing.

In systems representing the current standard of high-throughput flow cytometry, accurate quantitative measurements have been demonstrated in endpoint microassays at rates of 20–40 samples/min over a 4-decade range of fluorescence intensity using input cell concentrations of 1–20 million/ml and source well volumes of 5–15 μl [55]. Typical sample volumes of 1–2 μl allow scarce quantities of test cells or reagents to go a long way. Novel developments in sample delivery, data collection, and advanced analysis packages for processing algorithms, graphics, and multiplatform capabilities continue to add value to the utility of flow cytometry. Specific applications using flow cytometry-based multiplexing techniques are presented in detail in Section 5.

4. Fluorescence Technology

Fluorescence-based assays for high-density and high-content screening provide multidimensional readouts including intensity, lifetime, anisotropy, and spectral characteristics, each of which has been exploited by various analytical approaches that monitor changes in at least one of the fluorescence parameters. The use of fluorescence readout has some inherent disadvantages, most notably, autofluorescence and quenching from nonbiological interactions with the target. Each of these scenarios can also result in deterioration of other parameters such as fluorescence polarization.

4.1. Fluorescence-Based Assays

Measurement of integrated fluorescence intensity within a sample well, such as in flow cytometry-based assays, scales linearly with fluorescence quantum yield, and the major advantage of this approach, is straightforward detection and analysis. Fluorescent probes allow for the measurement of a wide variety of extrinsic cellular characteristics, and an expanding number of fluorescent probes is increasing access to a diversified set of cell-associated structures and physiological processes. A comprehensive discussion of the use of fluorescent probes as tools for flow cytometry has recently been published [65].

Flow cytometry is unique among fluorescence-based bioanalytical platforms in its ability to make simultaneous correlated optical measurements on individual particles at high rates. Exposure of a cell or a bead to excitation light occurs during a brief (a few microseconds) passage through an

illumination source in a nearly constant velocity flow stream. Under these highly uniform detection conditions and due to the extremely transient nature of fluorescence from organic molecules, flow cytometry is highly suitable for applications requiring precise measurements, such as DNA content between cells that may vary by only a few percent.

Modern flow cytometry is presently amenable to assay miniaturization down to 8 μl with the introduction of platforms such as HyperCyt®. In recent studies from our laboratory, we routinely analyzed up to 3000 cells from each sample well with an average sampling time of $\sim$1.5 s. HyperCyt® has been successfully adapted to cell-based end point assays [66], and studies investigating cell–cell adhesion [48, 67], and fluorescent ligand binding to cellular receptors [68, 69].

Fluorescence polarization utilizes linearly polarized light for excitation, and the emission is detected to infer changes in molecular orientation and mobility. Biological phenomena such as binding or cleavage will produce a change in polarization or anisotropy due to changes in mass or lifetime of the labeled analyte. Polarization readouts are limited by fluorescence quenching and autofluorescence and the range of molecular mass is limited by the fluorophore lifetime [70]. For example, protein–protein interactions have an upper limit of 50 kDa using fluorescein or rhodamine labels (fluorescence lifetime = 4 ns). Multiplexed fluorescence polarization assays have been described for identifying selective steroid hormone receptor ligands [71, 72], and for screening inhibitors for ribonuclease H activity of HIV-1 reverse transcriptase [73].

It is worthwhile to explicitly compare flow cytometry and fluorescence polarization assays. While both flow cytometry and fluorescence polarization are homogeneous modes of detection, they are remarkably different with respect to their experimental implementation. Generally speaking, fluorescence polarization assays require that both binding components be at concentrations in the vicinity of the dissociation constant of the binding interaction [70]. Under these conditions, a significant fraction of the fluorescent component will be bound to the nonfluorescent component. The fluorescent component in polarization assays is usually a small molecule whose molecular rotation is slowed by the interaction with the larger molecule. The size limit depends on the fluorescence lifetime of the probe, usually a few nanoseconds, which for practical reasons limits the size of the small molecule to $<$10 kD.

For flow cytometry, neither the lifetime of the fluorescence probe nor the rotation of the probe contributes to the detection sensitivity. Rather, detection has to do with observing fluorescent signals associated with the particle. The flow cytometer detects a pulse of fluorescence associated with the particle when there is bound fluorophore as compared with the fluorescence

associated with the solution surrounding the particle [65]. In microplate assays with 10 μl volumes, flow cytometry typically requires 1 pmol or less of the particle-associated assay component. Our experience suggests that it is preferable to associate the most precious component with the particle and that the quantity of material required is dependent on the affinity of the association with the particle rather than the affinity between interaction of the assay components.

4.2. Fluorescent Labels

For multiplexing applications, the practical limitations of using multiple, distinguishable fluorophores are simultaneously defined by the extent of overlap in emission spectra, the diversity of available excitation sources, compatibility with common optical filter sets, and the chemical properties of the dyes. A number of comprehensive technical reports and handbooks for the use of fluorescent probes are available from commercial suppliers [74, 75].

Fluorescein, a commonly used fluorophore, has high absorbance and high fluorescence quantum yield characteristics and exhibits good water solubility. The excitation maximum for fluorescein is closely paired with the 488 nm spectral line of the argon-ion laser and is therefore well-suited for flow cytometry. Amine-reactive fluorescein conjugates are widely used for labeling proteins. Disadvantages include photobleaching and pH sensitivity ($pK_a \sim 6.4$), and a broad emission spectrum that limits utility of the fluoresceins in multicolor experiments.

The rhodamine dyes are less sensitive to pH; however, their planar conformation leads to low water solubility and a tendency to dimerize with other labeled species. Low-molecular-weight cyanins and rhodamine derivatives absorb and emit at longer wavelengths compared with fluorescein, and are useful in combination for applications requiring dual color analysis [75].

The newer series of Alexa and BODIPY dyes include derivatives that span the visible spectrum. The Alexa series of dyes were largely developed to address the limitations of organic fluorochromes with regard to photostability, pH sensitivity, and stability of emission characteristics upon conjugation [76]. Alexa dyes have relatively high quantum yields and excitation maxima that closely match the wavelengths of commonly used excitation sources.

Semiconductor nanocrystals referred to as quantum dots (QDs) are robust bright light emitters that offer some advantages over organic fluorochromes, particularly for multiplexing applications [77]. Quantum dots exhibit size-dependent emission wavelength characteristics. Because multiple QD series can be detected by a single laser, multiparameteric analysis can be performed with single laser systems thus expanding the multiplexing abilities of the most basic cytometry instrumentation. The full impact of QD technology for

multiplex applications, however, is in polychromatic flow cytometry systems. In principle, QD colors and intensity combinations can be maximized to encode millions of analytes simultaneously [78].

The characteristically narrow and symmetrical emission spectra of QDs are particularly suited for multiplex applications that must be optimized by compensation to reduce spectral overlap using conventional fluorophores. Quantum dots can simultaneously be used with the common organic fluorochromes with no emission overlap or sensitivity compromise into the QD channels. Technical considerations and detailed compensation requirements for using QD/organic fluorochrome combinations are summarized in a number of technical reports on QDs [78–80].

5. Flow-Cytometry High-Content Profiling Applications

Performing multiple optical measurements on discrete particles carried in a sample stream is a traditional element of cytometry-based analytical approaches performed in populations of cells. While the flow cytometry platform has always had the analytical power for high-content multiparametric measurements, a correspondingly large-capacity analytical substrate format for multiplex was not available. Fluorescence-encoded microsphere sets were largely pioneered through the efforts of the Luminex Corporation. The technology provided the substrate for extending traditional cellular analysis into particle analysis while bringing the multiplexing concept of the planar microarray into suspension assays.

Multiplexed particle-based assays are now common in clinical diagnostics applications and are becoming increasingly appreciated for their utility in discovery research. Table 2 is a summary of commercially available bead sets that have been fully optimized for multiplex clinical and research applications.

5.1. Principles of Multiplexing for Suspension Arrays

The new fluorescence and bead technologies discussed in Sections 3.3 and 4.2., when paired with the inherent multiparametric features of flow cytometry afforded by the use of multiple lasers and highly resolved signals to multiple detectors, make this technology uniquely suited to multiplexing. The components that determine content analysis in multiplexing applications include the analytes, the addressing strategy, and how the interrogation is applied to produce the assay readout.

In contrast to spatially addressed planar arrays, suspension arrays are monitored by interrogation at optical addresses, specific to each reagent or

TABLE 2
MULTIPLEX BEAD SETS AND COMMERCIAL SUPPLIERS

Application	Suppliers
Adhesion Molecule Panels	R&D Systems
Allergy testing	BD Biosciences, ImTech
Apoptosis	BD Biosciences
Autoimmune markers (human, mouse)	Rules Based Medicine, Inc., SmartBead Technologies (Pronostics)
Cancer Markers	Rules Based Medicine, Inc.
Cardiac markers	Rules Based Medicine, Inc., Beckman Coulter
Cytokine profiling	Qiagen, BD Biosciences, R&D Systems, Bio-Rad, BioSource, LINCO, Upstate, Rules Based Medicine, Inc., Bioergonomics
Endocrine markers	LINCO, Rules Based Medicine, Inc.
Gene expression	BioSource, Marligen Biosciences
Genotyping/Genetic disease screening	MiraiBio, Marligen Biosciences, TmBioscience, Ambion Diagnostics, Tepnel Lifecodes, Illumina
HLA DNA typing	Tepnel Lifecodes, One Lambda
Infectious disease	Rules Based Medicine, Inc., Proactive Medical Technologies
Isotyping	Upstate, Rules Based Medicine, BD Biosciences
Metabolic markers	Rules Based Medicine, Inc, LINCO, R&D Systems
Phosphoprotein quantification	BD Biosciences, Upstate, BioSource
Ser/Thr Kinase	Qiagen
Transcription factors/nuclear receptors	Bios, Marligen Biosciences
Tissue Typing	One Lambda
Th1/Th2	Beckman Coulter, BD Biosciences
Bead sets for custom conjugation	Qiagen, Spherotech, Pierce, Bang's Laboratories, Polysciences, Inc.

bead set and fluorophore combination. The interrogation process in a multiplexed assay can address the capture components or the detection reagents. In assays where multiple reagents are coupled to the same bead, the corresponding ligands are coupled to unique fluorochromes. For low complexity bead sets using bead populations identified by unique fluorescent labels, an analyte-dependent signal is generated by detection reagents carrying a second type of signal. Larger populations of bead sets can be constructed from ratiometric staining with a combination of dyes and a signal generated by detection reagents coupled to a third type of dye [52].

The diversity and flexibility of bead-based suspension array technology offers a platform for high-throughput analytical approaches for a variety of applications that are reviewed in Sections 5.2 , 5.3, and 5.4. Cell-based assays

are also amenable to simultaneous multiplexing of several cell populations and high-content analysis of several features of display or response in each cell population.

5.2. Multiplexed Nucleic Acid Endpoint Assays

In the most straightforward nucleic acid analysis, the assay chemistry is followed by the readout or detection step. *In situ* amplification strategies present an opportunity to incorporate a detectable label into the immobilized target, in addition to increasing the abundance of analyte. Other systems couple the signal detection to the hybridization event, using structural or enzymatic approaches, to activation of a quenched fluorophore [81–84].

Luminex xMAP® is an example of a commercially available bead array platform [85]. The technology is based on internally dyed microspheres using two fluorochromes with spectrally distinct properties. Reporter molecules coupled to a third fluorochrome provide the detection signal for the biomolecular interaction on the microsphere surface. Nucleic acid detection chemistries commonly include direct hybridization of a labeled PCR amplified target to capture probe-bearing microspheres for each sequence.

Solution-based chemistries involve the enzymatic incorporation of a capture sequence that allows annealing to a complementary sequence on the microsphere. Alternatively, sequence-based enzymatic methods include sequence-discriminating DNA polymerases and DNA ligases, and processes such as allele-specific primer extension (ASPE), oligonucleotide ligation assays (OLAs), and single base chain extensions (SBCE). Single nucleotide discrimination is a common application for direct hybridization techniques that has recently been validated using solution bead-based technology in genotyping assays for polymorphisms. In a study of 21-plex and 34-plex assays for SNPs located near the ApoE locus, a total of 181 genotypes were determined and confirmed by sequence analysis [86]. The SBCE approach was used to analyze 20 multiplexed SNPs in 633 patient samples with greater than 99% agreement in genotype assignments to gel-based OLA results [87]. In the same report, the assay was converted to an ASPE format to overcome some of the technical difficulties of SBCE. Fifteen SNPs were characterized from 96 samples, totaling 1440 genotypes. The ASPE assay was 98.7% concordant with the OLA findings.

Genetic disease screening using a bead-based platform and ASPE assays has identified five mutations in the cystic fibrosis transmembrane regulator (CFTR) gene [88]. In another prospective study in 400 newborns, the genotypes of 27 mutations in CFTR were reported [89]. A multiplexed bead-based hybridization assay has been described for identifying a total of six mutations related to clinical manifestations of hypercoagulable states or aberrant

platelet phenomena and bacterial colonization of indwelling catheters [90]. In a study of acute lymphoblastic leukemia (ALL), a technique was described for combining multiplex PCR with direct bead-based hybridization in a single multiplex reaction to report seven fusion transcripts of chromosomal translocations occurring in ALL [91].

Multiplexed bead-based flow cytometry has been broadly applied to the identification, detection, and discrimination of bacterial, viral, and fungal pathogens. In a recent review [85] of the agents that have been studied with the xMAP® technology alone, eight references of studies conducted between 1998 and 2004 described bead-based multiplex platforms in the analysis of over 27 microbial pathogens.

Multiplexed bead-based analysis for the detection of combined viral and bacterial agents has been reported in assay panels for differential diagnosis among groups of diseases, including commonly occurring pathogens in children, sexually transmitted infections, and for blood bank screening of infectious agents [92]. Typing of human papillomaviruses (HPV) to discriminate between high- and low-risk genotypes in a single reaction has recently been reported using bead-based multiplexed assays and type-specific oligonucleotide probes. Up to 100 HPV types were assayed simultaneously, and all were detected with high specificity and reproducibility. Detection limits ranged from 100 to 800 pg of PCR product, and the technique was validated with conventional methods [93]. These examples have important implications for the utility of multiplexing platforms in large-scale epidemiological studies, particularly considering the fact that cytometry instrumentation is becoming more accessible in resource poor settings.

5.3. Multiplexed Protein Endpoint Assays

The ELISA has been the conventional assay standard for quantitative analysis of protein, and several readout modes have been adapted to this platform (fluorescence, chemiluminescence, and absorbance). Immunoassay sensitivity can be in the picograms per milliliter range depending on the affinity and specificity of the reagents, and since ELISA assays perform serial measurements in individual wells, cross-reactivity between reagents is not an issue. Automation-compatible microwell formats for ELISA and the related ELISpot assays have improved the throughput of the assay. A number of reports have evaluated the use of bead arrays with regard to performance equivalency to ELISA in cytokine analysis [94–96]. In these studies, the correlation in findings between multiplex bead-based studies and ELISA varies widely, but the majority of studies compare the techniques favorably.

Perhaps the greatest influence of multiplexed bead array approaches has occurred in the area of cytokine assays. Because cytokine profiles have been

used as readout systems for disease and therapeutic monitoring, immunologic profiling has a number of important clinical applications. Table 3 presents a summary of these and other multiplexed bead-based applications.

Multiplexing provides comprehensive profiles within a timeframe not possible with iterative assays requiring large amounts of assay material. In a recent study of a large population living in an area endemic for an intracellular parasitic disease, a multiplexed microsphere-based assay was used to quantify nine cytokines simultaneously and to evaluate disease progression associated with a common Th1/Th2 response paradigm [108]. In this and other infectious disease applications, cytokine profiling by economically and technically feasible means has the potential to enable large population studies of disease mechanisms.

TABLE 3
Flow Cytometry Multiplex Bead-Based Application

Application	Sytems/processes	Selected references
Antibody profiling	Serotyping, seroconversion, chronic disease monitoring, anti-viral antibodies, therapeutic monitoring, seroconversion, vaccine development	[59–61, 97, 98]
Epitope profiling	Virus typing, T cell phenotyping, epidemiologic surveillance	[16, 92]
Cytokine profiling	Inflammatory cytokines, drug efficacy monitoring, anti-tumor immune responses, biothreat monitoring	[13, 94–96, 99–102]
Nucleic acid analysis	Sequence detection (gene expression, detection of PCR products), single nucleotide polymorphisms, microRNA analysis, RNA–protein interactions	[33, 81, 83, 85–88, 90, 91, 93, 103]
Cytogenetics	Quantification of genomic copy number, gene rearrangement, allelic determination of leukocyte antigens	[104, 105]
Mechanistic studies, pharmacodynamic profiling	Protease inhibitor screening, phosphorylation profiling, cell signaling, ligand binding	[64, 68, 69, 106, 107]

5.4. Multiplex Assays of Molecular Assemblies

The potential of flow cytometry in the arena of molecular interactions is just beginning to emerge [64]. Numerous studies have already described the interactions of many classes of molecules where binding affinity, kinetics, and even enzymatic activity have been reported [109]. These approaches are now being extended into the domain of multiplexing.

Recently a novel system has demonstrated the first multiplex flow cytometry-based protease assay with sufficient selectivity for measuring specific protease activity for two different classes of proteases [106]. Using a bead-based assay capable of interrogating 12 individual substrates simultaneously, the findings have applications for pharmaceutical screening of protease inhibitors and proteases involved in disease states. One disadvantage of this approach is that the use of microspheres limits the substrate concentration to the micromolar range, requiring either a high-affinity protease or high concentrations of a lower affinity protease. If high-affinity substrates are constructed, the cost and/or time associated with assay development may be considerable. The time resolution capabilities of this assay make it an attractive format for resolving the kinetic steps of binding, catalysis, and enzyme release and rebinding phenomena.

In our laboratory, we have developed a novel and generalized approach to investigate G-protein–coupled receptor molecular assemblies that have multiplex implications [107]. We solubilized a fusion protein consisting of the β2-adrenergic receptor and green fluorescent protein (GFP) for bead-based flow cytometric analysis in two different formats. In one case, the β2-adrenergic receptor GFP fusion protein was bound to beads displaying a conjugated ligand, dihydroalprenolol. This format permits analysis of the K_d (dissociation constant) for the fusion protein binding to the ligand and, in competition with other β2-adrenergic receptor ligands, provides K_d values for agonists and antagonists. The other format involved beads displaying chelated nickel that bound purified hexahistidine-tagged G-protein heterotrimers. These beads were able to bind the binary complex of agonists with β2-adrenergic receptor GFP fusion protein. The dose-response curves of ternary complex formation between ligand, receptor, and G protein revealed maximal assembly for ligands previously classified as full agonists and reduced assembly for ligands previously classified as partial agonists [110]. These beads could be used in a mechanistic mode to show that guanosine 5′-3-*O*-(thio)triphosphate-induced dissociation rates of the ternary complex were the same for full and partial agonists. Taken together, these results suggested that the association rather than the dissociation of the signaling complex was what differentiated partial agonists from full agonists.

When performed simultaneously with color-coded beads, the two assemblies discriminated between agonist, antagonist, or inactive molecule in a manner appropriate for high throughput, small volume drug discovery. This is because the ligand beads were sensitive to the presence of all ligands, while the G-protein beads were sensitive only to agonists, and discriminated full and partial agonists. From a quantitative perspective, these studies show applications of flow cytometry for measurements of numbers of binding interactions per particle, the binding constants, and the rate constants, all in a homogeneous format.

These types of assemblies can be further generalized, not only to other G-protein–coupled receptor protein–protein interactions but also to other types of assemblies and other signal transduction components. Thus, we have described an application for other elements of signal transduction cascades such as protein kinases. In our experiments, two hexahistidine-tagged activin receptor like kinases (ALKs) were expressed in *E. coli*, purified and bound to nickel beads. A fluorescent kinase ligand that binds to the ATP binding site of these kinases with nanomolar affinity was developed. Binding of the fluorescent kinase ligand with kinase on the bead made the beads bright, and inhibitors decreased the brightness. A test panel of 17 nonfluorescent kinase inhibitors, spanning two orders of magnitude affinity for the kinases, gave K_d values for the kinases that correlated well with a fluorescence polarization assay. Results were obtained for the kinases in duplex using colored beads and an autosampler to send beads from a 96-well plate to a flow cytometer in a format suitable for high-throughput screening.

Another multiplex application has been developed as a collaboration for the NIH Roadmap Molecular Libraries Initiative to study the Bcl-2 family of proteins, which in part governs apoptosis. The human genome contains six genes that encode anti-apoptotic Bcl-2 family members. Each of these proteins can be bound to endogenous proteins that contain a conserved peptidyl domain, the Bcl-2 homology region 3 (BH3). Pro-apoptotic family members include both multidomain proteins, including Bak, and "BH3-only" proteins, including Bim. These pro-apoptotic BH3 peptides that dock at this site in Bcl-2 and Bcl-XL also increased apoptosis of leukemia and lymphoma cells in culture and in Severe Combined Immunodeficiency (SCID) mice [111–113]. The binding of a fluorochrome-conjugated BH3 peptides to Bcl-2 family proteins thus provides the basis for the construction of fluorescence assays, suitable for high-throughput screening. Our collaborators have described two fluorescent peptides, F-Bim and F-Bak, that bind to six and four members of the Bcl-2 family, expressed as GST fusion proteins, respectively. The team has already devised procedures for producing multi-milligram quantities of purified recombinant proteins and devised a generic fluorescence polarization assay, using F-Bim [114]. They have performed a preliminary screen of

~10,000 compounds with Bfl-1 and F-Bim, demonstrating the suitability of this homogeneous assay for the high-throughput environment. Using these reagents and observations as a starting point, we have developed a multiplex analysis by HyperCyt® high-throughput flow cytometry where all six members of the family can be screened simultaneously, using color-coded beads displaying glutathione. These beads have been produced with high-surface density of glutathione that appears to stabilize the interaction of the proteins with the bead surface and allow the construction of the mixtures of the multiplexed assays [115]. We are currently working on suspension arrays for other families of proteins. For example, we have succeeded in displaying GST fusion proteins of low-molecular-weight G proteins that bind fluorescent derivatives of GTP.

The experimental conditions for flow cytometry require that one component be attached to a particle. As mentioned previously, it is advisable to build the assay so that the more precious component is associated with the particle. Typically, the association is through an epitope tagging scheme, likely to be the same one that was used in the purification of that assay component. The less expensive component is then provided at concentrations near the K_d of the binding interaction. Thus, one component is displayed at a concentration of tens of thousands of molecules per particle, on thousands of particles, in volumes of a few microliters. We typically use a picomole of protein or less per assay when the assays are performed in multiwell plates, in total quantities that can be hundreds of times lower than, for example, a fluorescence polarization assay. In addition, in some cases, we have been able to perform flow cytometric analyses without purification of the protein at all [116]. In this case, the protein of interest is captured on the flow cytometry bead by epitope recognition. Rather than removing the protein from the bead for further purification, it is sometimes possible to display it directly for subsequent interaction with the fluorescent component. Experience suggests that this may be more applicable when the fluorescent component is purified as would be the case for the binding of a fluorescent ligand to a protein displayed on a bead.

5.5. Throughput

The throughput of multiplex assays in flow cytometry is worth considering. Under optimal conditions, flow cytometers are capable of detecting tens of thousands of events per second. To a first approximation, the data acquisition capabilities are largely insensitive to the number of parameters that are detected. Thus, at one extreme, the flow cytometer could detect 10 or more parameters for each particle, functioning in a high-content mode. The prime example of this type of assay is to detect simultaneously many individual signals such as the levels of multiple phosphoproteins in cells [117].

Moreover, flow cytometry is equally adept at performing both high content and multiplex simultaneously. A well-known example of this type of application is where multiple antibodies are used to discriminate immunological diverse populations of white blood cells [2]. Here, the information content as well as the multiplex information is encoded by the antibodies. If there are additional colors that are available for detection, they may be used to measure the cell responses to a stimulus, for example the concentration of intracellular calcium, in individual cell populations.

In the high-throughput mode, multiplexing is subjected to different limitations. At one end of the spectrum, the limitation comes from the number of addresses in a suspension array that can be encoded. With four log decades of sensitivity in a typical flow cytometer, it has been practical to encode 10 color levels and 10 addresses. With two-color encoding, 100 addresses have been achieved. With a sampling rate of 10,000 events per second and 200–500 events per address for adequate sampling statistics, the sampling time for a 100-plex suspension array is theoretically only 2–5 s, with a flow rate of 1 μl/s and a particle density of 10,000 per μl. We have observed that it is entirely practical to sample 20-plex bead populations with a sampling time of $\sim$1 s on the HyperCyt® platform. The sampling time is expected to increase linearly with the multiplicity of the array. On the other hand, for complex molecular interactions, the biology of the assembly is more likely to limit the multiplicity of the array because of the limited specificity of the reagents rather than the technological aspects of sampling or detection.

6. Future Directions

The application of multiplexing to functional genomics or proteomics is now beginning to shift into high gear [118]. The potential will be recognized in the areas of protein–protein, protein–ligand or small molecule, and protein–RNA/DNA interactions. While there are perhaps 10 times as many proteins expressed as genes, when posttranslational modifications and splice variants are accounted for, it is likely in the foreseeable future that specific families of proteins, protein domains, or the protein-binding elements of RNA or DNA will be arrayed for multiplexing. These arrays are expected to probe family relationships involving specificity and affinity of the molecular interactions, rate constants for their assembly and disassembly, and discovery of small molecules. These small molecules will be used as probes for the molecular assemblies, as leads in drug discovery for therapeutics, and potentially as imaging agents for diagnostics.

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IMMUNE MONITORING OF CLINICAL TRIALS WITH BIOTHERAPIES

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1. Abstract

Immune monitoring of biotherapy clinical trials has undergone a considerable change in recent years. Technical advances together with new insights into molecular immunology have ushered a new genre of assays into immune

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monitoring. Single-cell assays, multiplex profiling, and signaling molecule detection have replaced formerly used bulk assays, such as proliferation or cytotoxicity. The emphasis on immune cell functions and quantitation of antigen-specific T cells has been playing a major role in attempts to establish correlations between therapy-induced alterations in immune responses and clinical endpoints. However, this has been an elusive goal to achieve, and there is a special need for improving the quality of serial monitoring to ensure that it adequately and reliably measures changes induced by administered biotherapy. In this respect, monitoring performed in specialized reference laboratories operating as good laboratory practice (GLP) facilities and strengthening of interactions between the clinical investigator, the clinical immunologist, and the biostatistician are crucial for successful use of immune monitoring in clinical studies.

2. Introduction

A wide variety of biologic agents from cytokines to dendritic cell (DC)-based vaccines have been used in clinical trials for patients with cancer, infectious diseases, or autoimmune syndromes. In general, the current interest in the clinical application of biologics to therapy of human diseases reflects the need for more effective, less toxic, and preferably natural ways of treatment, especially with conditions that are refractory to standard therapies. Biologic agents as therapeutics aim at a modification of biologic responses, often of the host immune system, to improve the host capability to recover and reacquire normal homeostasis. Because most biologics target molecular and cellular immunologic pathways, there is frequently a requirement for assessments of the direct or indirect effects they exert *in vivo*. Hence, immunologic monitoring has slowly emerged as an advisable adjunct to clinical trials with biologic agents. By and large, immunologic monitoring is not a protocol-mandated requirement but rather falls under the "correlative" studies category. This is because in phase I and phase II trials, safety/toxicity or clinical efficacy are the primary endpoints, respectively, and immunologic or biologic responses to therapy are considered as secondary endpoints. Nevertheless, immune monitoring is important for, ideally, it may be able to relate clinical responses to a specific immune mechanism, to predict subject responsiveness to therapy, or even to help estimate disease free or overall survival. The possibility that immune measures may serve as biomarkers or as surrogate endpoints of clinical responses has recently gained in popularity.

The immune system is exquisitely well prepared to handle insults by pathogens and to control autoimmunity [1, 2]. It is ready to respond to "danger" originating within or outside the body [3]. In pathologic conditions,

the immune system becomes compromised, even dysfunctional, and many biologic therapies attempt to restore its integrity and upregulate immune surveillance. Some biologic therapies target innate or natural immunity in hope of upregulating surveillance functions of the immune cells, such as monocytes, natural killer (NK) cells, or NKT cells [4]. Other biologic therapies preferentially aim at enhancing adaptive immune responses to selectively target those T or B lymphocytes that are responsible for protection against specific insults [5, 6]. Much has been learned about the immune system and its dysregulation in disease; however, the complexity of cellular and molecular interactions within the system, especially between innate and adaptive immunity, and the existing crosstalk with neural, endocrine, and hormonal networks complicate the interpretation of signals generated as a result of particular immune-based therapies. It is, therefore, a foregone conclusion that the interpretation of immune monitoring results will be difficult, often intuitive and not always informative.

In recent years, many sophisticated technologies have been introduced for measuring immune responses at the population as well as single-cell level. Older ''bulk'' assays have been largely replaced by single-cell assays, especially in measurements of antigen-specific T-cell responses. Capabilities exist for assessment of immune reactivity *in situ*, that is, at the tissue site of disease, as well as in the lymph nodes or peripheral circulation. Technologies currently available for measuring immune responses of patients enrolled in biotherapy trials are numerous and sophisticated. This includes high-throughput technologies such as arrays, multiplex formats, proteomics, genomics, high-content screening by flow cytometry, imaging, or tissue microarrays (see Table 1). Most of these technologies originated in research laboratories, and today most are not validated for use in monitoring. Immune monitoring of patients participating in clinical trials represents a drastic departure from the research

TABLE 1
TECHNOLOGIES AVAILABLE FOR ADAPTATION TO IMMUNE MONITORING OF PATIENTS TREATED WITH BIOTHERAPIES

1. Genomic analysis: DNA arrays
2. RT-PCR for molecular markers of disease
3. Serum/plasma and tissue proteomics, including antibody microarrays and multiplexing for cytokines and chemokines
4. Immune polymorphisms
5. High-content screening by flow and imaging cytometry
6. Tissue microarrays and immunocytochemistry
7. Assessment of immune infiltrates into tissues
8. Assessments of cell apoptosis vs. necrosis

environment. A technology or a method that works well in a research laboratory has to undergo a stringent scrutiny and considerable refinement in order to meet criteria for its use in monitoring. While the new "state-of-the-art" technologies offer possibilities for rapid screening of multiple samples and for profiling (i.e., simultaneous detection) of many immune biomarkers, none had been formally validated. This means that the application of these technologies to assessments of patient samples represents clinical research that has not yet achieved acceptance as monitoring. These technologies are mentioned but not discussed in detail here, and the reader is referred to the summary of a Workshop on Cancer Biometrics [7] for an excellent review for their use and potential.

The purpose of this chapter is to describe requirements for the process of immune monitoring, starting with selection of methods that are applicable to reliable serial assessments, continuing with their performance under defined quality assurance (QA) or quality control (QC) conditions, and ending with recommendations for results interpretation. The entire monitoring enterprise requires support that can only be provided in a specialized laboratory operated to handle and reliably test serial specimens and, preferably, functioning as a good laboratory practice (GLP) facility.

3. Rationale for Immune Monitoring

"Monitoring" refers to serial specimen acquisition and testing. The rationale for immune monitoring rests on the premise that therapeutic interventions achieve their effects as a result of modification(s) in one or more components of the patient's immune system. These therapy-induced modifications occur gradually, and the expectation is that by serially measuring immune biomarkers that undergo changes relative to the pretherapy baseline level, it might be possible to define immunologic mechanisms responsible for biologic and possibly also clinical activity of the therapeutic agent. As biologic agents have a bell-shaped activity curve that shifts depending on the dose and time of their delivery, serial monitoring is necessary to define the optimal biologic dose (OBD) of a therapeutic agent, which often is distinct from the maximal tolerated dose (MTD). The latter is utilized to define toxicity of drugs, but because most biologic agents have no or little toxicity, the OBD is the appropriate measure of their effects. Since, however, biologic agents are likely to have multiple biologic (and clinical) effects, the definition of OBD may not be straightforward, depending on more than one immunologic assay. The major objective of serial immune monitoring is to establish a correlation between phenotypic and/or functional changes in immune cells induced by therapy and clinical responses. The major unanswered question,

however, concerns the origin of immune cells to be tested. Peripheral blood mononuclear cells (PBMC) representing less than 2% total body mononuclear cells are most commonly employed, although it appears that cells derived from the disease site (e.g., site of infection, tumor, tumor-draining lymph nodes, fluid from an inflamed joint, or interstitial fluid from injured sites) best reflect the extent of alterations induced by disease. Thus, whenever available, such specimens should be collected, banked, and evaluated in parallel with peripheral blood.

3.1. Requirements

A brief description of requirements that underlie the principles of immune monitoring is provided to orient the reader. Specimens collected from subjects prior to, at defined intervals during, and at the end of therapy are delivered to the laboratory. The specimens usually consist of peripheral blood collected into heparinized tubes, but may include tumor or other tissues, body fluids (e.g., pleural or peritoneal fluids, ascites), as well as especially collected interstitial fluids from sites of cannulation [8]. The specimens for immune monitoring are harvested at intervals specified in the clinical protocol and have to arrive at the laboratory no later than 24 hours after harvest. This requires an overnight delivery of specimens originating at a distant location. The specimens are bar-coded and processed immediately upon arrival. The separated cells are either cryopreserved at -80 °C in 2 ml cryovials and banked or are immediately tested in assays that cannot be performed with cryopreserved/thawed cells. The monitoring laboratory is cognizant of assays that have to be performed on fresh as opposed to cryopreserved/thawed cells and will be prepared to handle the specimens accordingly.

Changes occurring in the immune cell phenotype or function in response to therapy may be difficult to detect, unless sensitive and reliable monitoring assays are available. To decrease interassay variability of assays, immune monitoring is generally performed with "batched" specimens, representing the entire collection of samples obtained from one subject throughout therapy. This type of design mandates that all collected specimens be cryopreserved under controlled conditions, thawed with a minimal loss of viability, and tested in the same assay. It also requires that the monitoring laboratory has the capability to perform cryopreservation, bank, and maintain samples at a large scale for prolonged periods of time. An assay "reliability" in this context depends on the selection for monitoring of those immune markers/functions that are least affected by cryopreservation/thawing. This has to be a priori ascertained by the monitoring laboratory through comparisons of fresh and frozen specimens tested in the same assay. Experience

shows that the correctly performed process of freezing/thawing of immune or other cells is by far the most crucial determinant for preserving their true functional potential and, hence, for successful monitoring.

3.2. Significance

Immune monitoring of clinical trials is a complex and demanding enterprise requiring considerable resources. Its translational role in bridging basic immunologic insights with clinical endpoints, however, cannot be overemphasized. There is increasing awareness of the fact that biologic therapies occupy an important place among available clinical modalities for treatment of human disease. Their true impact on disease processes cannot be unraveled without a better understanding of immune mechanisms that these therapies target and possibly alter. Without reliable immune monitoring to help identify and define these mechanisms, biotherapy is unlikely to achieve a strong scientific foundation it deserves. Additionally, immune biomarkers identified by well-done monitoring might well prove to be significant surrogates of disease development, activity, or progression, and as such play a key role in clinical practice. Immune monitoring represents a valuable component of future research in translational science and clinical medicine.

4. Selection of Assays for Immune Monitoring

Technical advances and new insights into immunologic mechanisms have led to the recent development of many new types of immunoassays that lend themselves to use in monitoring (Table 2). Today, clinical investigators have a choice between phenotypic vs. functional, specific vs. nonspecific, and direct vs. indirect immune assays. The range and sophistication of currently available assays, which can potentially be used for monitoring, predicate that careful consideration is required for selection of the assay that best fits with the hypothesis being tested and with the laboratory expertise. It is essential to remember that immunologic monitoring of serially collected and batched samples is more demanding and rigorous than the performance of individual research assays. To monitor credibly, the laboratory has to have methodology in place that is selected for optimal performance and consistently provides reliable results. Thus, the selection involves a consideration of the assay attributes, including its throughput, required equipment, cost, and the expertise level necessary for its routine performance. Occasionally, a clinical investigator may request an assay that is especially applicable to a given clinical protocol or is newly available and thus interesting. In all instances, it is critical to select an assay that can accurately measure therapy-induced

TABLE 2
ASSAYS CURRENTLY AVAILABLE FOR IMMUNE MONITORING OF BIOTHERAPY TRIALS[a]

Assay type	Sample type
Phenotypic markers	
Cells: T, NKT, B, NK, M, DC, PMN, tissue cells	
Absolute cell numbers	Whole blood, body fluids, tissue biopsy
Cell proportions (percentages)	Whole blood, body fluids, tissue biopsy
Cellular subpopulations	Whole blood, body fluids, tissue biopsy
Single-cell quantification	Isolated cells
Morphology, cytology	Isolated cells
Functional assessments	
Delayed-type hypersensitivity (DTH)	Skin test read at 48 hours
Proliferation	MNC, tissue cells, isolated cell subsets
Cytotoxicity (CTL, ADCC, NK, LAK)	MNC or isolated cell subsets
Suppression (treg)	MNC or isolated cell subsets
Migration (chemotaxis)	Isolated cell subsets
Signaling, signal transduction	Tissue biopsy, isolated cells
Superoxide generation	MNC, tissue cells, tissue biopsy
Enzymatic activity	MNC, tissue cells, tissue biopsy
Apoptosis or necrosis	MNC, tissue cells, tissue biopsy
Cellular products	
Immunoglobulin levels	Serum, plasma, body fluids, supernatants
Cytokine/chemokine levels	Serum, plasma, body fluids, supernatants
Cytokine receptors (soluble)	Serum, plasma, body fluids, supernatants
Other soluble surface molecules (e.g., HLA, β_2m)	Serum, plasma, body fluids, supernatants
Ligands (e.g., FasL, TRAIL) and growth factors	Serum, plasma, body fluids, supernatants
Enzymes (e.g., metaloproteinases, arginase, IDO)	Serum, plasma, body fluids, supernatants
Neopterin	Serum, plasma, body fluids, supernatants

[a] The assays listed are available to be individually adapted to immune monitoring for phase I/II clinical trials. Many are not commercially available, and their use as an immunologic endpoint in phase III clinical trials would require formal validation.

changes in the frequency and/or function of immune cells. The selected assay should be easily adaptable to serial testing with a minimal loss of accuracy, have a high throughput to accommodate large-volume testing, and lend itself to automation. These requirements are imposed by the nature of monitoring which has to accommodate batched serial samples from many time points collected on protocols that enroll multiple subjects. The investigator and the laboratory personnel are generally required to devote considerable time and effort to assay selection, as the decision may determine not only scientific/correlative results of testing, but may also have significant financial impact. Therefore, selection of a monitoring assay is an important step with consequences that are likely to affect results emerging from a clinical trial and, ultimately, will have impact on the field of biotherapy as a whole.

4.1. Use of Cryopreserved vs. Freshly Harvested Specimens

The first decision facing a monitoring laboratory is the selection of assays that can be performed with cryopreserved cells or frozen samples of body fluids without compromising cellular functions or analyte integrity, respectively. In monitoring, serially collected specimens are processed, cryopreserved/frozen, and batched for testing upon thawing. This is by design because the only reliable way to determine differences between pretherapy and posttherapy results is to test these samples in the same assay. Consequently, the ability to reliably bank body fluids or cryopreserve immune cells for testing at the time therapy is already completed is the key to success. However, certain immunologic assays, notably those that measure cytotoxicity, cannot be reliably performed with cryopreserved cells [9]. It is necessary to compare fresh and cryopreserved/frozen specimens to ascertain that a test gives the same results when performed in parallel with either type of specimen. Assays accompanied by documentation showing they are reliable when performed with cryopreserved/thawed specimens are the best candidates for serial monitoring. Conversely, assays that must be performed on freshly harvested samples require documentation of interassay variability so that therapy-induced changes in immune parameters can be distinguished from assay-related spurious differences.

4.2. Single-Cell Assays: Tetramers, CFC, ELISPOT

In subjects with cancer treated with therapeutic antitumor vaccines, three single-cell assays for detection of antigen-specific effector T lymphocytes can be used for immune monitoring: enzyme-linked immunospot (ELISPOT) assay cytokine flow cytometry (CFC), and tetramer binding. Each assay is capable of measuring the frequency of vaccine-specific or tumor-specific T cells in a mononuclear cell specimen. All three have been rapidly replacing bulk-culture assays, such as cytotoxicity (i.e., ^{51}Cr-release assay), proliferation (i.e., ^{3}[H] thymidine incorporation), or cytokine production. All three are based on T-cell receptor (TCR) recognition of cognate peptides presented by MHC class I or class II molecules on the surface of antigen-presenting cells (APC) to the responder T cells. However, no consensus exists as to which of these three assays should be selected to best monitor vaccination results. The common perception that these assays are equivalent, that is, provide the same results, is not correct. Using all three assays, we measured the frequency of the melanoma peptide-specific CD8+ T cells in the peripheral circulation of subjects with metastatic melanoma who had received multiepitope DC-based vaccines [10]. A concordance between the three assays was determined using a 3×3 scatter plot matrix design constructed for each of the four peptides tested in all three assays before and after

vaccination therapy was completed. The three single-cell assays were not found to be concordant in measuring the frequency of immune effector cells in the peripheral blood of vaccinated subjects. The results for tetramer staining were consistently higher than those obtained with the ELISPOT or CFC assay [10].

Reflecting upon the observed data and the assay characteristics, it becomes clear that result concordance should not be expected. The ELISPOT assay measures production of cytokines (most commonly either IFN-γ or IL-5) by individual T cells in the plated population with a theoretical detection sensitivity of 1/100,000 cells. CFC identifies single responding T cells (1/50,000) with a cytokine expression in the Golgi zone. Tetramer binding detects peptide-specific T cells expressing the relevant TCR with a theoretical detection sensitivity of 1/10,000 cells. The assays not only have different sensitivities of detection, but also differ in specificity. ELISPOT and CFC are antibody-based and, by definition, are highly specific. In contrast, tetramers, which are complexes of peptides sitting in grooves of four MHC molecules held together by a streptavidin-biotin scaffold [11], bind to T lymphocytes expressing the relevant TCR with variable affinity. Tetramers might easily dissociate or nonspecifically bind to B cells, monocytes, or apoptotic cells [12, 13]. Thus, tetramer specificity needs to be carefully controlled. While separation of relevant (e.g., CD8+ or CD4+) T-cell subpopulations by antibody-charged columns or gate dumping of irrelevant cells in flow cytometry enhances tetramer-binding specificity, these procedures are costly, labor/time consuming, and thus not readily applicable to serial monitoring. Further, T cells that bind tetramers may not be functional, as TCR signaling could be compromised, as often happens in cancer or chronic infections, such as HIV-1 [14, 15]. This reduces the tetramer-binding assay to a phenotypic category because it detects T cells that bind tetramers but are not always functional [16]. CFC measures cytokine expression in a cell and not its secretion (although it is commonly assumed that the expressed cytokine would be secreted) and, strictly speaking, is also not a functional assay. Before flow cytometry, responder T cells are incubated for a few hours with a stimulant in the presence of Brefeldin or Monensin to block cytokine secretion [17]. While necessary, this step allows for undesirable amplification of response, as interactions in culture might increase the frequency of responding cells in the tested population. Cell permeabilization necessary for intercellular staining of a Golgi-based cytokine might introduce problems with immunodetection in CFC assays. ELISPOT is based on the similar principle as CFC, only it measures cytokine *secretion* from stimulated responder cells that are plated as a monolayer of individual cells on a nitrocellulose membrane to avoid cell-to-cell contact and allow for adequate spot display. Only ELISPOT measures function of individual responder cells by identifying those that produce and secrete the measured cytokine. ELISPOT also

does not require cell permeabilization or the use of a flow cytometer for cytokine detection.

4.3. ELISPOT As a Monitoring Assay

Which of these three assays is to be selected for immune monitoring of antigen-specific T-cell frequencies in a clinical trial? This frequently asked question can now be rationally answered as follows. Because it is always preferable to measure function rather than phenotype, ELISPOT would be an assay of choice. However, CFC and tetramer-binding assays are flow cytometry-based and thus allow for surface labeling of responder cells and their identification. It is possible to select CD8+ or CD4+ T-cell subsets on antibody-charged columns prior to ELISPOT [18], and two-color ELISPOT now available offers a possibility of identifying T cells simultaneously producing two cytokines [19]. In addition, supernatants from ELISPOT wells can be collected and tested for cytokine levels in multiplex assays. On the other hand, tetramer binding can be combined with both surface staining to determine cellular phenotype and intracytoplasmic staining for the detection of cytokine production [16]. While most informative, especially in situations when some of tetramer-binding cells do not express cytokines, this rare-event analysis is time-consuming, labor-intensive, and thus, not the best choice for serial monitoring. The recommended solution would be to monitor by ELISPOT or CFC (but not both), depending on considerations, such as sample numbers, time, labor, cost, and access to a flow cytometer. Tetramer binding could be used as a confirmatory assay in situations where it is important to demonstrate a functional deficiency of tetramer-binding T cells. The fact that tetramers have to be custom-made and pretitered for every peptide further limits their application to monitoring. In the author's experience, ELISPOT performed under strictly controlled, preferably GLP, conditions is most likely to provide accurate estimates of the frequency of functionally competent effector T cells in batched, serial samples obtained from subjects enrolled in clinical studies. Compared with CFC and tetramer binding, the cost of ELISPOT is reasonable enough to permit its use in a high-volume testing. However, the ELISPOT assay is not easy to standardize, and responder–stimulator interactions might result in unacceptably high background spot counts in which case the assay becomes uninterpretable.

4.4. Attributes of Selected Assays

Selection of a "right" assay for immune monitoring is a difficult process. An assay that accurately records therapy-induced changes in one or more immune parameters may not always be applicable to high-throughput serial

testing. The single-cell assays described above are complex in execution, difficult to analyze and interpret, and are relatively costly. It follows that their use should be judicious and carefully planned. Whatever assay is selected, it should have most, if not all, of the following attributes when performed under defined standard conditions: specificity, sensitivity precision, accuracy, and robustness. However, immunologic assays are often cell-based and thus especially troublesome to standardize, as discussed below, and may not be as precise or robust as chemical assays. For example, it is not unusual to see CVs greater than 20% in cellular assays. Functional assays are likely to be more difficult and expensive to perform than phenotypic assays. Cellular assays are always more complex to execute and interpret than titrating or measuring concentrations of soluble analytes in body fluids. Assay standardization, which is a requirement for serial immune monitoring, is expensive and requires special expertise. Ultimately, an investigator faced with a choice has to make an informed selection based on the hypothesis to be tested and monitoring tools that are most likely to accomplish the task.

5. Profiling of in Immune Biomarkers

Immune responses involve cascades of balanced interactions between antigens, immune cells, and their products. An imbalance or perturbation of the immune network may be more readily detectable if several rather than one functional or phenotypic markers are measured. Technologic advances now allow for a simultaneous assessment of multiple biomarkers, providing an investigator with a "profile," which may be more informative than a single assay. For example, cytokines and chemokines, soluble products of immune cells, are known to function as networks of several biologically related mediators, and measurements of a single cytokine in biologic fluid are seldom informative. The preference for simultaneous definition of several mediators contributing to an immune response has led to the development of microarrays and multiplex assays.

5.1. Multiplexing for Cytokines

Cytokine, gene, or protein profiling, whether by multiplex immunoassays, microarrays, or proteomics technologies, is especially well suited to evaluations of cytokine circuits. The potential for capturing polarization in the cytokine repertoire or differences in patterns of their production by immune or tissue cells and of relating them to a specific clinical situation has a tremendous appeal. Systemic and local therapies with cytokines are becoming increasingly common, and there is a need for monitoring cytokine levels in

relation to clinical endpoints. It is expected that such monitoring will expand our knowledge of the cytokine biology and rapidly provide a wealth of clinically useful information about cytokine involvement in human disease.

Today, multiplex assays have all but replaced traditional enzyme-linked immunosorbent assays (ELISA) for cytokines allowing us to simultaneously measure proinflammatory cytokines, Th1- vs. Th2-type cytokines, growth-promoting as opposed to suppressive cytokines, and so on, in a small (0.5 ml) sample of body fluid. Multiplex bead immunoassays designed to work in conjunction with a Luminex-type instrument utilize sets of distinct fluorescently labeled microspheres, each covalently linked to a cytokine-specific antibody [20, 21]. A combination of different color-coded beads (up to 100) in one tube allows for a simultaneous assessment of several cytokines. A flow-based instrument equipped with a reporter and classifying lasers and associated optics measures reactions that occur on the surface of colored microspheres. A high-speed digital signal processor efficiently manages the fluorescent output. The intensity of measured fluorescence is directly proportional to the concentration of the cytokine present in the specimen. The result is a quantitative profile of as many as 20–30 cytokines that might be especially useful for assessments of cytokine imbalance in disease. The therapeutic goal of shifting the balance from Th1 to Th2 cytokines can be more clearly defined using "cytokine profiling." Specifically, in the Th1-dominant diseases characterized by excess production of such Th1 cytokines as IL-2 or IFN-γ (i.e., autoimmunity, graft vs host disease), the desirable outcome would be a shift toward a Th2 cytokine profile. Conversely, in cancer, HIV or allergy considered to be Th2-dominant diseases, with excess of IL-4, IL-5, IL-10 production, a therapeutically driven shift toward the Th1 profile might correlate with immune and perhaps clinical recovery [22, 23]. It has been suggested that measuring cytokines and other growth factors in serum is not as useful as profiling them *in situ*, at the disease site. To this end, a technology exists collecting interstitial fluids via special catheters to be tested for multiple cytokines/chemokines by a microassay method, as recently described [8].

The multiplex antibody-coated bead assays for cytokines are referred to as "cytokine arrays." Several other assays utilizing a microarray format for rapid detection of cytokine profiles in biologic samples are available as recently reviewed [24, 25]. Their advantages are that numerous cytokines/chemokines can be simultaneously detected in a small sample volume with a high throughput.

5.2. Genomics and Proteomics

Microarray formats can of course be applied to measuring proteins other than cytokines including peptides, small molecules, or metabolic by-products present in body fluids. Under the heading of proteomics, these technologies

are currently used to define biomarkers that would qualify as "surrogate markers" of disease and provide a signature of disease state. It is expected that pathologic changes on the one hand and therapy-induced alterations on the other in cells or tissues will be reflected in distinct protein patterns detectable in cells or body fluids. Proteomic technologies that are able to discriminate between normal and pathologic protein patterns are likely to become very useful for mapping changes that accompany therapy or are associated with a disease progression. Proteomic methods involve protein quantification and their characterization, including posttranslational modifications or protein–protein interaction profiles [26, 27].

Although proteomics-based screening for biomarkers is commonly a component of clinical trials with biologic agents, its application to monitoring will depend on highly validated and characterized methods of sample analysis. Similarly, assessments of gene expression profiles using array-based technologies, referred to as genomics and widely used today in efforts to provide a genetic phenotype of a disease state, are not yet ready for use in monitoring. Nevertheless, both genomics- and proteomics-based technologies are gaining increasingly important place in screening for immune alterations and will likely become major components of monitoring in the future, provided current correlative studies validate their reliability and clinical utility [7, 28, 29].

Detection of differences in gene expression, using microarrays now widely available from commercial vendors, in immune cells prior to and after therapy is likely to help in identifying molecular targets responsible for a response. Similarly, a combination of two-dimensional PAGE with mass spectrometry allowing for simultaneous analysis of many hundreds of proteins in body fluids or tissues may enable us to discriminate disease-associated from therapy-induced effects. Rapid and cost-effective screening for the expression of multiple genes coding for cytokines, activation markers, components of major signaling pathways, or other cellular products offers a powerful new approach to obtaining an integrated view of disease mechanisms and cellular processes. Specifically, measuring T-cell activation, or identifying changes in T-cell differentiation, proliferation, and survival in response to biotherapy represents an important advance in understanding molecular mechanisms of immune response. In addition, examining up- or downregulation of gene expression as well as protein analysis capturing distinct protein patterns could identify biomarkers of prognosis or surrogate markers correlating with clinical responses. These potential applications of genomics and proteomics to monitoring of research clinical trials are being actively explored. But to the best of the author's knowledge, none of these "state-of-the-art" technologies have yet been qualified for monitoring of biotherapy trials based on the *feasibility*, *reproducibility*, and *standardization* criteria usually applied to assays selected for diagnosis, early detection, monitoring of therapy, or prevention and risk assessment.

5.3. Polychromatic Flow Cytometry

The availability of monoclonal antibodies labeled with various chromatophores with excitation wavelengths spanning the entire light spectrum has revolutionized flow cytometry. This technology is used for examining cells, including immunocytes, in body fluids or in culture. The presence and/or levels of expression of surface or intracytoplasmic markers in immune cells can be reliably assessed by flow cytometry. Newer applications of flow to measuring cellular functions such as proliferation, cytotoxicity, apoptosis, or cytokine expression are replacing more traditional culture-based methods [30–32]. Single-cell assays, such as tetramer binding or CFC, as discussed above, allow for examination of individual antigen-specific immune cells and their quantification. Further, this multiparameter technology facilitates simultaneous analysis of the phenotype and function and lends itself to high-content screening [33].

Flow cytometry-based assays are now widely accessible and are highly accurate. A large array of variously labeled monoclonal antibodies are commercially supplied, allowing for the assessment of the proportions of immune cells positive for up to 9–12 markers. A combination of activation markers, growth receptors, cytokine receptors, costimulatory molecules, and so forth, on distinct and identifiable subsets of PBMC provides a powerful tool for monitoring. However, from the practical viewpoint, monitoring for simultaneous expression of more than five markers is too labor-intensive, especially in its data analysis aspect, and in the author's monitoring laboratory four-color flow cytometry assays are routine, while flow with five or more colors is used for confirmatory/research purposes. Changes not only in proportions of cells expressing a given set of markers but also in levels of expression of individual molecules or even changes in their phosphorylation following exposure to an activating signal can be quantified by flow cytometry. This feature makes multiparameter flow cytometry a useful approach for testing the predicted mechanisms of action of a biologic agent. Quantitative flow analysis can be greatly enhanced by the use of molecular equivalents of soluble fluorochrome (MESF) units instead of mean fluorescence intensity (MFI) in every assay. By adding a mixture of four types of beads with known fluorescence intensities and unlabeled blank beads control, a standard calibration curve is generated each time the assay is performed. The curve transforms MFI values into MESF units and defines the interassay variability of the assay (e.g., $<2\%$ with $n = 30$). This method is particularly useful for assessments of individual cellular components such as activation markers or signaling molecules in lymphocytes. Phenotyping of cells by flow cytometry can be done on fresh or cryopreserved specimens, and it has been extensively used in monitoring of biotherapy trials. However, calibration of the instrument and

compensation for chromatic interference are the key to successful and reliable flow cytometry.

6. The QA/QC Program and Assay Quality

An assay selected for use in monitoring has to be executed under conditions that will consistently provide acceptable data. Immune monitoring of patients enrolled in biotherapy trials should only be performed in laboratories with established QA and QC programs that meet the GLP standards. QC of immunologic assays, especially cellular assays is difficult and that of serial immunologic measures represents a special challenge. Therefore, a rigorously operated QA/QC program is a requirement for a monitoring laboratory. Such a program generally consists of several components, including a manual of regularly updated standard operating procedures (SOP), policy manual, personnel training, instrument maintenance, reagent and temperature control, secure data base, adverse event review, quality review, and proficiency testing. Currently, no model QC program exists for immunologic monitoring, and the laboratories are encouraged to follow the GLP guidelines offered by professional groups, such as College of American Pathologists or the departments of health in some states. No proficiency testing programs are available for most immunologic assays, except those designed for flow cytometry or hematology (i.e., the leukocyte count and differential). Participation in these is highly recommended. Largely, however, the monitoring laboratories are required to establish their own QA/QC program to ensure that acceptable data are generated in compliance with the GLP standard. In cases where this is not possible, services of a reference immune monitoring laboratory can be sought. Advantages of a central laboratory operated as a GLP facility are listed in Table 3. Such facilities exist in large medical centers or may be associated with National Institutes of Health (NIH-) supported cooperative groups.

TABLE 3
ADVANTAGES OF A CENTRAL LABORATORY OPERATED AS A GLP FACILITY

- QA/QC program in place, assuring the quality and reliability of monitoring
- State-of-the-art technologies in use
- Scale-up and high-throughput assays available
- Assay development and standardization
- Decreased cost of monitoring due to the large assay volume
- Result interpretation by a clinical immunologist in conjunction with statisticians aware of immune-based analyses
- Banking of samples which are accompanied by clinical outcome data for future research

6.1. QA/QC in Practice

Sample arrival, log in, and processing performed according to an existing SOP have to be organized to meet the specimen collection schema(s) in a clinical protocol(s). Arrival of serial specimens is recorded manually and electronically. Blood samples for immunologic monitoring should be routinely collected in the morning to avoid diurnal variability. Tissue samples and body fluids are processed differently than blood, and the laboratory has to be prepared to handle these specimens as they arrive. Specimens must reach the laboratory within 24 hours of collection to be processed. A history of each sample is maintained, and problems are documented in writing and reviewed. Arriving specimens are directed to be tested fresh or cryopreserved. The decision to use fresh or cryopreserved cells in a given assay is made prior to a clinical protocol initiation and is based on results of preliminary comparative studies of the assay performance with fresh vs. cryopreserved cells. Cryopreservation, using a rate-control freezing device, is a routine but extremely important step in monitoring as is banking of cryopreserved specimens in liquid N_2 vapors. Both are performed under stringent controls because subsequent testing depends on the quality of cells that are stored frozen and thawed for testing. Similarly, aliquoted body fluids that are banked and batched for future testing must be protected from temperature fluctuations or inadvertent equipment failure. Hence, temperature-control program has to be in effect around the clock. An automated temperature-control system is advisable. The above-described activities are components of the QA/QC program that is necessary for reliable immune monitoring.

6.2. Assay Standardization

Standardization is performed prior to the introduction of an assay for monitoring. As the major function of serial monitoring is to document changes from baseline upon treatment, the importance of assay reproducibility cannot be overemphasized. The standardization data are obtained by repeatedly performing the assay with cells or body fluids obtained from healthy donors under previously established optimized invariant conditions to establish the mean, median, and 80% normal range and coefficient of variation. The intraassay variability is also determined, as batching of specimens in one assay is a usual operating procedure. A set of appropriate controls is selected, and these depend on the type of specimen. For example, in assays performed with fresh PBMC, which are collected at various time points, interassay variability is of concern. Therefore, a cryopreserved lot of vialed PBMC obtained from a normal donor can be prepared, its range

of reactivity determined, and thawed cells used in the assay to monitor day-to-day variability. With fresh cells, it is always advisable to include fresh control cells obtained from a healthy volunteer. In this respect, it is necessary to have in place an IRB approval for drawing blood from consented normal donors and to maintain a pool of such donors for QC purposes.

Batching of serial samples obtained from one patient in the same assay may dispense with concerns of interassay variability; however, even with this monitoring strategy in place, it is necessary to control for day-to-day differences to ensure the assay performs equally well for all patients on the protocol, whose batched specimens are likely to be tested on different days. The data obtained from control samples and evaluated in parallel with each patient sample can help to ensure the results validity. Whenever available, universal standards (such as e.g., cytokine standards available from NIH or WHO) should be regularly included in the monitoring assays.

6.3. Assay Validation

Assay "standardization" should not be confused with assay "validation." The former is a requisite part of the QC program, while the latter is a formal evaluation of an assay performed as defined in the Bioanalytical Method Validation issued by FDA in May 2001 (21CFR part 58) and qualifying it to be used in phase III clinical trials as an immunologic endpoint. Validation consists of a series of experiments designed to evaluate accuracy, precision, selectivity, sensitivity, reproducibility, and stability characteristics of the method. While validation criteria are well defined for chemical assays, immunologic assays do not always fit into these criteria. Nevertheless, because no separate guidelines exist for immunologic assays, those developed for chemical assays are generally followed. Validation of immunologic assays is expensive and faces a number of problems, especially with cell-based assays, which are typically lacking in precision. Further, no reference standards for cellular assays are currently available. In contrast, validation of immune assays measuring soluble products, such as immunoglobulins or cytokines, can follow recommendations for the development of chemical assays.

7. Interpretation of Immune Monitoring Assays

Serial immune monitoring is aimed at the accurate identification of changes in the immune profile established at baseline during the course of therapy. Not unexpectedly, there are many pitfalls. Even when the measurements of samples collected over time are made simultaneously and

accurately in one assay, it is possible to miss the change, especially when it peaks, simply because sampling intervals are not correct, so that the relevant samples are missing. A separate concern is that of biologic variability existing within and among individuals. Immune responses are subject to environmental, hormonal, and neurologic as well as pathologic changes [34, 35]. Therefore, the discrimination of therapy-related alterations from biologically mediated normal responses to infections, stress, or endocrine activity might be very difficult. Often, this might require a parallel assessments of nonimmunologic biomarkers.

7.1. Statistical Analysis

Results obtained from serial monitoring require statistical analysis. In preparation for such analysis, the data have to be purged of possible errors made during data entry. A monitor intimately familiar with the assays is assigned the task of screening for outliers. These are identified and checked against the laboratory records (worksheets). Next, the statistician responsible for the analysis should generate a summary data statement in which missing data points or variations from the protocol schema are flagged to be checked against the laboratory records. This is a very important component of the analysis because changes in the timing of samples relative to the treatment as well as departures from the sampling sequence specified in the protocol are likely to have profound effects on the final results. Once the statistician is satisfied with the completeness and accuracy of the data set, he initiates the analysis that he had selected together with the investigator responsible for the clinical trial. The selected analysis depends on the trial objectives, its design, and hypotheses tested, but since pre- and post-treatment changes are generally measured, the object of the analysis is to determine the significance of changes from pretherapy baseline. Several statistical methods are available for this type of analysis as previously described [36].

The statistician performing the analysis of immune monitoring results has to be intimately familiar with the assay(s) that had been used. This is often a problem, which can, however, be easily remedied by including the clinical immunologist in the design, execution, and analysis of biotherapy clinical trials which include immune monitoring. Interpretation of immunologic data is not straightforward: it requires knowledge of the assays performed as well as the biologic agent used for therapy and its mode of action. It is necessary to be cognizant of the hypothesis being tested in the trial and putative mechanisms that might mediate immunologic activity or its absence, as when, for example, suppressive mechanisms counterbalance stimulatory activity of a therapeutic agent. For this reason, immunologic results might

appear counterintuitive, and expertise is needed to provide a correct interpretation. Individuals who direct immunomonitoring laboratories are in a position to help with assays selection, their timely and accurate execution and, by being in touch with the statistician, also with the data analysis. Close interactions between the biostatisticians, clinical investigator, and the laboratory are hallmarks of successful monitoring.

7.2. Interpretation Discrepancies

Given the stringent requirements and demands good monitoring imposes, it is not surprising that to date few significant correlative studies have emerged from immune monitoring. Further, immunologic results obtained in various academic centers testing similar or the same immune therapies rarely agree. The data interpretation becomes challenging when two different assays give results that do not correlate. For example, delayed-type hypersensitivity (DTH) test, which is the only available *in vivo* correlate of cellular immunity in man often does not correlate with results of *ex vivo* T-cell assays. A positive DTH response read as induration (not erythema) at the test site 48 hours after intradermal application of an antigen signifies existing immunity [37]. A concomitant absence of proliferative *ex vivo* T-cell response might be due to the assay sensitivity and thus may not be biologically significant. Another test, for example, cytokine production, might be positive. On the other hand, a negative DTH test accompanied by a positive *ex vivo* response to the same antigen could indicate *in vivo* suppression that is not detected with isolated T cells in a test tube. When results of two *ex vivo* assays do not correlate, the tendency is to depend on one and neglect the other. However, the observed discrepancy could signify a loss/gain of a specific immune function in which case an important biologic event is missed. More relevant to monitoring is the situation when changes from the baseline only weakly correlate with a clinical endpoint or when large individual variability in the magnitude of immune response exists among subjects. The latter is a common finding, and the use of robust fitting statistics might correct for between-patient differences. In some individuals, immune response to treatment may be delayed relative to others, and here also statistical modeling might simplify interpretation. Overall, while discrepancies are expected between the DTH and *ex vivo* assays, those between two monitoring *ex vivo* assays are troublesome. It is, therefore, preferable to avoid monitoring of the same function, for example, cytotoxicity, using two different assays. Monitoring based on the hypothesis which targets a specific immune mechanism or activity should be restricted to one, at most two, carefully selected assays. Interpretation of results from such a hypothesis-driven study will be simpler, and results are more likely to correlate with clinical outcome. On the other

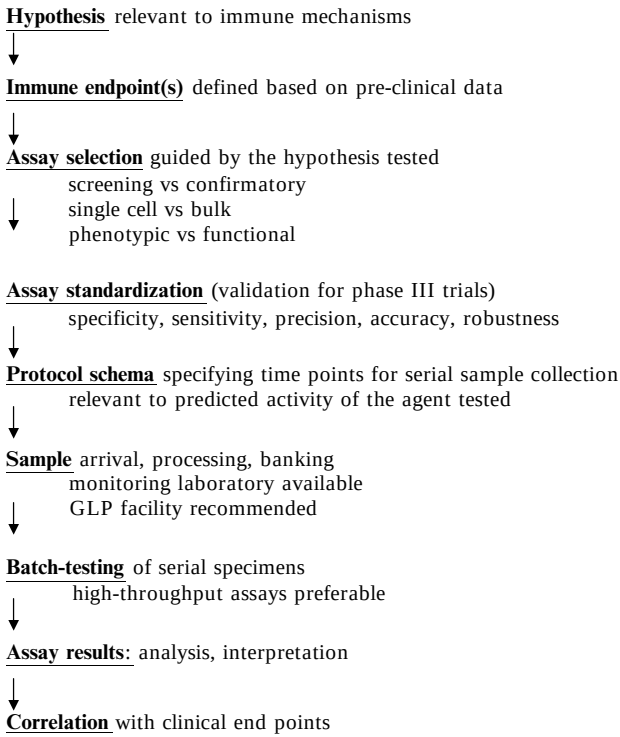
hand, currently popular microarrays, serving as screening tools, may identify biomarkers that correlate with clinical endpoints or can be used as surrogate markers. When achieved, this would represent a significant advance in monitoring. As always, the availability of data from adequate numbers of subjects together with the ability of a selected monitoring assay to discriminate responders from nonresponders will determine the level of significance. It should be remembered that both can be and should be determined prior to the clinical trial based on the available preliminary data.

8. Conclusions

Immune monitoring of serially collected samples from clinical protocols is demanding, resource consuming, and costly. It is, however, necessary for ensuring that clinical endpoints are correlated to the immunologic mechanisms potentially responsible for therapy-induced changes. Linking correlative immunologic studies with clinical endpoints, however, has been a major problem. One reason could be that requirements for extensive laboratory support in the context of existing QA/QC program have not been met. Serial studies are especially difficult to perform reliably, and a GLP facility specializing in immune monitoring is an appropriate venue for such studies. A schema for immune monitoring as it should be optimally performed in support of clinical trials is included below. While there are many steps, the selection of assays that are most likely to capture the underlying immune mechanisms is of critical importance. Yet, this is not a simple task because interactions between immune cells, cytokines, antibodies, and other biologically significant immune products and the host are not fully understood. Interpretations of monitoring results require statistical analysis capable of modeling therapy-induced changes in multiple cellular interactions over time. It is expected that when these requirements are adequately addressed and immune monitoring becomes an integral part of all biotherapy trials, the so far tenuous goal of establishing immune correlates of clinical responses will be fulfilled.

The future of immune monitoring includes introduction of new assays targeting signaling molecules, activation pathways, apoptosis antigen processing, and regulatory T lymphocytes (Treg). Also, antigen-specific responses will be playing an increasingly important role and will become validated to meet regulatory requirements. Immune measures are beginning to be looked upon as biomarkers of disease progression and outcome. This trend is likely to continue given the emerging evidence that phenotypic and functional attributes of immune cells as well as their products can serve as surrogate markers of disease progression or responses to therapy. Multiplex formats

A schema for immune monitoring of clinical trials



and microarrays are slowly replacing conventional immune assays and allow for the definition of immune profiles, which appear to be more informative and biologically relevant. With a better understanding of immune mechanisms and their involvement in human disease, it will be possible to eliminate the practice of using multiple assay panels and replace it with the hypothesis-driven monitoring dependent on one or a small number of selected assays. Most of these alterations in the practice of monitoring are already in progress, and in its new format, immune monitoring is well poised to continue serving the field of biotherapy.

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DIETARY MODIFICATION OF BRAIN FUNCTION: EFFECTS ON NEUROENDOCRINE AND PSYCHOLOGICAL DETERMINANTS OF MENTAL HEALTH- AND STRESS-RELATED DISORDERS

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1. Abstract

Stress is associated with both psychological and biological adaptation. Chronic stress, however, impairs adaptation, and may finally lead to illness, in part through unhealthy changes in nutritional behavior. This chapter shows how physiological and psychological stress responses are affected by

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different food ingredients, and how stress affects health behavior, for example food choice. It becomes obvious that nutrition is closely linked to food choice and that food ingredients affect a broad range of neuroendocrine and related psychological processes, which regulate adaptation to chronic stress. Thus, dietary modification may become a valuable tool to modify the susceptibility to stress and stress-related disorders.

2. Introduction

Stress may affect health not only through its direct biological effects but also through changes in health behaviors [1, 2]. One such health behavior is food choice. Stress may lead to illness through unhealthy changes in nutritional behavior. In psychology literature, food choices have often been considered as one of the range of health-related behaviors that might be responsive to life stress or emotional well-being either inadvertently or as a deliberate strategy for coping with stress [3, 4].

Stress is associated with biological changes, such as adrenaline-induced glycogenolysis, slowed gastric emptying, autonomic shunting of blood from gut to musculature, and activation of the hypothalamic–pituitary–adrenal (HPA) axis [5, 6]. Increases of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol levels in anticipation of or during stressful stimulation are interpreted as allostatic [7]. The diversity of health implications now associated with control and consequences of the release of cortisol, together with its sensitivity to psychological stress, has given this major human adrenal glucocorticoid (GC) hormone much importance in behavioral medicine [8]. There is evidence that the HPA axis as well as peripheral cortisol metabolism may be differently regulated according to sex and age factors [9, 10].

The HPA axis is closely associated with systems responsible for caloric flow in the body [11–13]. Only recently animal studies have revealed evidence for fasting-induced attenuation of pituitary-adrenal responses to stress. A prolonged fasting of 4 days decreased cortisol responses to stress in sheep isolated from the flock compared with stressed sheep fed *ad libitum* [14]. Rats fasted for 14–24 hours showed a blunted corticosterone response to novelty [15] and reduced ACTH levels following restraint stress compared with controls [16, 17]. However, despite lower ACTH levels, restraint stress after fasting was associated with an increased corticosterone response in the latter experiments [17]. In humans, fasting induced changes in adrenocortical responsiveness [18]. Fasting for 3 days led to reduced cortisol increases following insulin-induced hypoglycemia. Fasting for 24 hours was without any effect (Fig. 1).

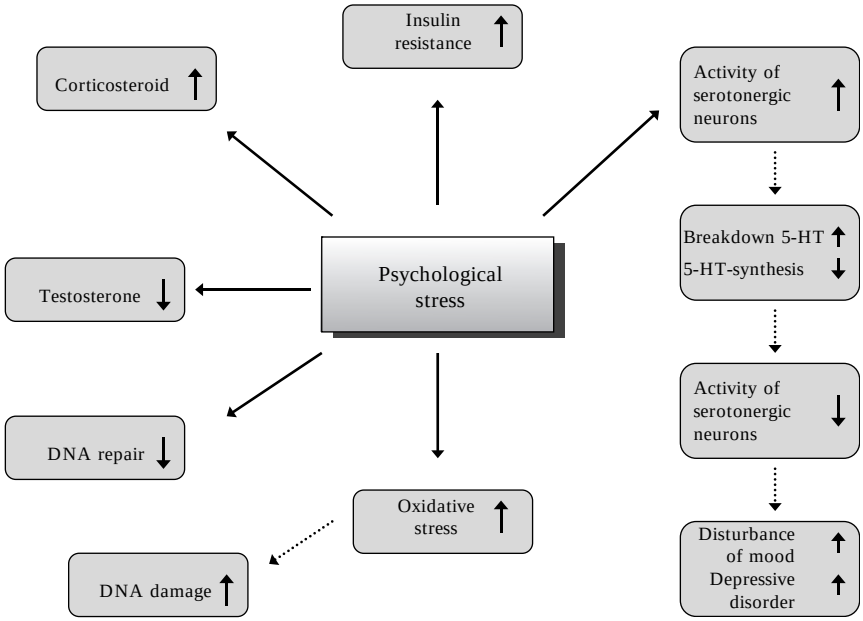

FIG. 1. Physiological effects of psychological stress.

HPA axis function can profoundly influence expression of appetite and regulation of body weight [6], whereas HPA axis activity can be modified by changes in feeding patterns [19]. Systematic studies demonstrated an association between meals and the midday increase in plasma cortisol [20]. Also, increases in plasma cortisol could occur in some subjects after breakfast, the midday meal, or an evening meal, the greatest effect occurred after the midday meal [21].

3. Meal Composition

Food ingestion is a well-known inducer of several peptides that may, in turn, directly influence the activity of the HPA axis [22]. Animal studies indicated that both the food intake and the light–dark cycle represent independent synchronizers for the circadian periodicity of cortisol secretion [23]. In addition, meal timing, food composition, and the duration of premeal fasting have been shown to exert an important effect on cortisol secretion [24, 21]. Cortisol secretion elicited by stress or smoking is abolished by mild food deprivation in men [12]. Normal physiological variations in cortisol in

humans have a significant direct influence on macronutrient metabolism [25, 26]. Cortisol seems to increase lipolysis and proteolysis, as well as increasing gluconeogenesis, thereby raising the contribution of protein and fat to energy substrate supply, while protecting glycogen stores. Further, the ability of cortisol to increase plasma free fatty acid (FFA) levels may underlie the emerging link between cortisol and abdominal obesity, together with its associated metabolic syndrome [27].

In humans, it has been found that food ingestion, particularly proteins, can stimulate α1-adrenoreceptors, possibly via the activation of neurotransmitter amines [28]. There is much evidence that adrenal steroids influence macronutrient selection by increasing appetite for carbohydrates (CHOs), primarily, and for fat, and regulating the timing of eating in rodents [29, 30]. Also, stress may alter macronutrient selection. Women tend to prefer high-fat or sweet foods when moderately stressed [31, 32]. Women who were high cortisol reactors to stress ate more food than low reactors during recovering from stress [33]. On the rest day, however, high reactors tended to eat less and low reactors tended to eat more, eliminating the difference between groups. The high cortisol reactors tended to consume more sweet foods than low reactors, across days [33]. Also cortisol reactivity may be a marker for vulnerability to stress-induced eating, and thus may help to explain who eats more vs who eats less after stress [33].

3.1. Carbohydrate

Diets with high CHO content may prevent deterioration of mood in stress-prone subjects when submitted to a stressful task [34]. Different studies indicated that CHO supplementation results in significantly lower plasma levels of tryptophan and the branched-chain amino acids (leucine, isoleucine, and valine) by 120 min of exercise [35, 36]. The rise in plasma tryptophan levels is believed to be the result of attenuation in FFA levels as a result of the CHO supplementation. Increasing levels of plasma FFA result in increased plasma levels of tryptophan by displacing tryptophan from albumin [36, 37]. In contrast, declines in plasma branched-chain amino acid levels during CHO supplementation and exercise are thought to be due to the maintenance of plasma insulin levels during exercise [37, 38].

Studies indicated that CHO supplementation results in lower plasma levels of both amino acids tyrosine and phenylalanine. Since movement of these amino acids into muscle and liver can also be enhanced by insulin [36, 39]. CHO supplementation also influences the hormonal responses to exercise. Also, CHO supplementation during prolonged exercise attenuated increases in plasma cortisol and decreases in plasma insulin [35, 40]. The magnitude of decline in plasma insulin levels during CHO supplementation appears to be

greater in women compared with the decline previously described in men [35]. Glucose load leads to a rapid rise in insulin levels in nondiabetics, and thereby to an increased transport of tryptophan into the central nervous system. This is followed by an increased synthesis of serotonin, which is known to have a stimulatory influence on the HPA axis at the hypothalamic level [41]. Following Trier Social Stress Test subjects with high blood glucose levels showed the well-established response pattern of a twofold increase in free cortisol levels [42, 43]. Human studies suggest that ready availability of energy is a prerequisite for significant acute stress responses of the HPA axis [12].

3.2. Fat

Feeding rats chronically a high-fat diet increased their basal and stress-induced HPA activity [44]. Continuous high-fat feeding may act as a chronic stressor, not only enhancing baseline adrenocortical activity but also increasing neuroendocrine stress responses. Feeding rats with high-fat diet resulted in a lower anxiety level in an elevated plusmaze paradigm compared with feeding with high-CHO diet [45]. Feeding rats a diet with a high fat content reduces some of the behavioral and physiological responses to psychosocial and physiological stressors such as social defeat and administration of the endotoxin lipopolysaccharides [46]. Furthermore, the defeat-induced desensitization of central nervous 5-hydroxytryptamine (5-HT)1A receptors, which normally occurs in animals on a diet with a high CHO content, is absent in animals on a high-fat diet [46]. Hypothalamic levels of 5-HT also decrease immediately after consumption of a fat meal [47]. A high-fat diet has suppressive effects on the thermoregulatory and behavioral responses to stress [46]. Serum brain-derived neurotrophic factor has been found to be negatively correlated with the severity of depressive symptoms [48]. Brain-derived neurotrophic factor expression can be inhibited by physical and psychological stress [49] and a diet high in saturated fat and sucrose [50, 51].

It has been suggested that dietary fat is a prime contributor to the development of obesity [46]. For any given body mass index, mortality is higher if fat is distributed centrally (visceral adiposity) compared with a more generalized pattern of distribution [52]. This has renewed interest in the factors that control adipose tissue distribution in addition to adipose tissue mass and function [53]. Although men tend to progressively increase abdominal fat deposits with increasing total adiposity at each age, a tendency to develop different obesity phenotypes throughout the lifespan occurs more clearly in women, particularly after the menopausal age [54]. The response of the HPA axis to a high-lipid/protein meal or high-CHO meal in obese women depends on their pattern of body fat distribution and that the activation of

the HPA following the ingestion of large amounts of complex CHO may have some pathophysiological relevance, specifically in women with the abdominal obesity phenotype [55]. In obese women, different mechanisms may be responsible for the regulation of the HPA axis following a high-lipid/protein meal or high-CHO meal, depending on their phenotype [55]. It is well known that catecholamines are involved in the regulation of the HPA axis [56]. Animal studies on norepinephrine turnover have shown that food intake stimulates sympathetic nervous system activity [57].

3.2.1. *Monounsaturated Fatty Acids*

In vitro addition of physiological concentrations of exogenous FFAs confirms that the stimulation of chromogranins B (CGB) binding properties is mainly due to monounsaturated fatty acid (MFA) classes. Oleic acid alone mimicked the *in vivo* situation by increasing the affinity constant of CGB for cortisol (threefold) and reducing the number of binding sites (twofold), whereas saturated fats did not enhance the binding [58]. Addition of monounsaturated FFAs to purified human CGB enhanced CGB binding activity in a concentration-dependent fashion [59].

3.2.2. *Polyunsaturated Fatty Acids*

Polyunsaturated fatty acids (PUFAs) are long-chain *n*-3 and *n*-6 fatty acids (FAs) of plant and marine origin. These essential FAs cannot be synthesized in the human body. Due to their greater availability and low cost, there is excessive consumption of n-6 FA in developing countries. Junk food is also loaded with n-6 FAs and trans-FAs. The ideal ratio of n-3 to n-6 PUFA is $\sim$1:1, according to the conclusion of an international panel of lipid experts [60]. The biological importance of PUFAs derives in part from their role as precursors of important second messengers (prostaglandins, prostacyclins, and leukotrienes) [61, 62] and as constituents of structural lipids in cellular membranes, which influence the activities of membrane-linked functional molecules (receptors, enzymes, and transporters) [63, 64]. The presence of large amounts of n-3 PUFAs in the brain is indicative of the major role that these compounds play in the structure and function of this organ [65]. Fish provide varying amounts of n-3 PUFA in the form of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). There is a significant negative correlation between worldwide fish consumption and prevalence of depression [66]. Frequent fish consumption in the general population is associated with a decreased risk of depression and suicidal ideation [67]. Further, fish consumption is significantly associated with higher self-reported mental health status [68]. Also, higher concentrations of DHA in mother's milk and greater seafood consumption both predicted lower prevalence of postpartum depression [69]. A number of investigations have found a decreased

n-3 PUFA content in the blood of depressed patients [70–73]. In fact, EPA content in red blood cells phospholipids is negatively correlated with the severity of depression, while the ratio of n-6 PUFA arachidonic acid to EPA positively correlates with the clinical symptoms of depression [70]. In addition, a negative correlation between adipose tissue DHA and depression has been observed. Mildly depressed subjects had 34.6% less DHA in adipose tissue than nondepressed subjects [74].

n-3 PUFAs are an essential component of central nervous system membrane phospholipidacyl chains and as such they are critical to the dynamic structure of neuronal membranes [75]. DHA is continuously secreted by astrocytes, bathing the neuron in n-3 PUFA [76]. The binding of serotonin to the astroglial 5-HT2A receptor can mobilize DHA to supply the neuron [77]. Alterations in membrane lipids can alter function by changing fluidity. An optimal fluidity is required for neurotransmitter binding and the signaling within the cell [78]. n-3 PUFA can alter neuronal fluidity by displacing cholesterol from the membrane [79].

Chronic dietary deficiency in α-linolenic acid impairs performance in learning ability and motivational processes [80]. Also, reduction in n-3 PUFA intake results in a reduction of n-3 PUFA content throughout the brain cells and organelles along with a compensatory rise in n-6 PUFA acid content. This alteration is accompanied by a 40% reduction in the Na/K-ATPase of nerve terminals, an enzyme that controls ion transport produced by nerve transmission and that consumes half the energy used by the brain [65]. A 30% reduction in the average densities of synaptic vesicles in the terminals of the hippocampal CA1 region has also been observed as a result of an n-3 PUFA deficiency combined with a learning task [81]. Deficiency of n-3 PUFA also results in a 30–35% reduction in phosphatidylserine concentration in the rat brain cortex, brain mitochondria, and olfactory bulb [82].

n-3 PUFA deficiency induces changes at several levels of the dopaminergic mesocortico limbic pathway [83]. In animal studies, n-3 PUFA-deficient diet resulted in a reduction of the dopaminergic vesicle pool [84] along with a 40–60% decrease in the amount of dopamine in the frontal cortex and an increase in the nucleus accumbens [84, 85]. Further, n-3 PUFA deficiency in rats reduced the release of dopamine from the vesicular storage pool under tyramine stimulation by 90% than in receiving an adequate n-3 PUFA intake [84] (Table 1).

The amount of n-3 PUFAs in the diet might act on the regulation of cerebral gene expression [83, 87]. Addition of PUFA to purified human CGB enhanced CGB binding activity in a concentration-dependent fashion [59]. Garrel *et al.* [88] showed that CGB and cortisol levels did not indicate any changes by the consumption of n-3 and n-6 FAs.

TABLE 1
CONSEQUENCES OF OMEGA-3 DEFICIENCY IN THE CENTRAL NERVOUS SYSTEM [86]

Parameters showing decrease	Parameters showing increase
1. Blood–brain barrier integrity	1. Dopamine content in nucleus accumbens
2. Dopamine content in frontal cortex	2. Pre/postsynaptic dopamine receptor DR2 in nucleus accumbens
3. Dopamine vesicle pool	3. Serotonin receptor (5-HT2) density in frontal cortex (compensatory response)
4. Fluidity at surface polar membrane	
5. Glucose uptake by neurons	
6. Hippocampal CA1 pyramidal neuron cell body size	
7. Neuronal cytochrome oxidase activity	
8. Normal cerebral microperfusion	
9. Normal inhibitory control over nucleus accumbens dopamine	
10. Phosphatidylserine in cortex, olfactory bulb, and mitochondria	
11. Pre/postsynaptic dopamine receptor DR2 in frontal cortex	
12. Na/K-ATPase at nerve terminal	
13. Vesicular monoamine transporter	
14. Dopamine release from vesicle storage	

Clinical trial demonstrated that 4 months of treatment with 9.6-g n-3 PUFA can be of benefit in the treatment of bipolar disorder. Also, n-3 PUFA had significant effect in lengthening remission and in treating depression [89]. A double-blind, placebo-controlled study ($n = 22$) showed that the addition of 2 g EPA to standard antidepressant medication enhances the effectiveness of that medication compared with the medication plus placebo after 3 weeks of treatment. EPA had an effect on insomnia, depressed mood, and feelings of guilt and worthlessness [90]. In a 12-week, randomized, double-blind, placebo-controlled trial, patients who experienced persistent depression, despite ongoing standard pharmacotherapy received 1 g EPA. The patients showed a 53% reduction on Hamilton depression scores. Intake of 1 g EPA dose led also to improvements in depression, anxiety, sleep, lassitude, libido, and suicidal ideation [91].

3.3. PROTEIN

There is some evidence that maintaining a very high-protein diet may chronically stimulate the HPA axis [92] and increase release of vasoactive hormones [93]. Increased HPA activity and cortisol release have been linked

to increased risk of insulin resistance, hypertriglyceridemia, and hypercholesterolemia [94, 95]. Hypercholesterolemia can be caused by high intake of animal protein [96]. Reducing the CHO:protein ratio of diets chronically has also been associated with deterioration in mood [97, 98]; this deterioration may be caused through poor acceptability of the foods by subjects [99].

After a protein-rich meal (30–40% energy as protein) salivary cortisol increases substantially (approximately one-and-a-half- to twofold on average). The increase begins toward the end of a 30-min meal period and peaks at ~1 hour after the start of the meal. After ~2 hours, salivary cortisol levels declined and were no longer significantly different from those seen either in the absence of a meal or after a low-protein meal [100]. Greater than 5% protein (as percentage total energy) is required to detect a reliable increase in salivary cortisol [100]. Results of studies measuring plasma cortisol suggest that at least 10% protein may be needed for reliable stimulation of cortisol [101]. A meal intake of at least 20 g of protein may be necessary; moreover, the higher the intake of protein, the greater the secretion of cortisol is likely to be [100, 101]. This acute stimulation of cortisol release may be part of a homeostatic mechanism in response to a high influx of amino acids [100].

Previous studies indicated that postmeal plasma cortisol levels could be affected by the proportions of macronutrients consumed. Meals containing 20–40% protein (as percentage total energy) produced a greater plasma cortisol response than meals with high CHO or fat levels [102]. By comparison, meals containing 10% protein resulted in weaker secretion of plasma cortisol [101], and protein-free glucose or fat loads did not stimulate cortisol release [23]. There is some evidence that maintaining a very high-protein diet may chronically stimulate the HPA axis [92] and increase release of vasoactive hormones [93].

3.4. Antioxidant

Oxidative stress is one of the most important factors mediating the deleterious effects of aging on behavior and neuronal function [103]. The central nervous system appears to be especially vulnerable to the effects of oxidative stress, partially as a result of additional factors such as increases in the ratio of oxidized glutathione to total glutathione [104], significant lipofuscin accumulation [105] with B-cell leukemia/lymphoma 2 increases [106], increases in membrane lipid peroxidation [107], reductions in glutamine synthetase [108], reductions in redox-active iron [105, 109], and alterations in membrane lipids [110].

Most dietary agents used to alter behavioral and neuronal effects with aging included nutritional supplements such as vitamins C and E, garlic [111], herbal supplements (e.g., ginseng, *Ginkgo biloba*, and ding lang) [112].

Long-term (from 6 to 15 months of age) feeding of F344 rats with an AIN-93 diet supplemented with strawberry or spinach extract (1–2% of the diet) or vitamin E (500 IU) retarded age-related decrements in cognitive and neuronal function compared with an AIN-93 diet alone. Results indicated that the supplemented diets could prevent the onset of age-related deficits in several indices (e.g., cognitive behavior and Morris water maze performance) [113].

3.5. Phytochemicals

Fruits, vegetables, and common beverages as well as herbs and plants have been shown to be rich sources of microchemicals with different healthy effects. They are most effective at ameliorating age-related deficits (e.g., signal transduction, motor performance, and cognitive behavior) [114]. Food chemists and natural product scientists have identified hundreds of phytochemicals, for example, carotenoids, chlorophyll, flavonoids, and sulfides. They have the potential, for example, to modulate stress. Persons who eat green or yellow vegetables everyday show a lower incidence of stress syndrome (irritation, sleeplessness) than those who do not eat them daily [115].

Human studies indicated that β-carotene suppresses the secretion of CRH dose dependently [116]. It is also suggested that the effective site of β-carotene is the hypothalamus, where β-carotene suppressed the secretion of CRH induced by exercise stress, and consequently the secretion of ACTH in the pituitary. As CRH stimulates the sympathetic neuron [117], β-carotene also inhibited the stimulation of noradrenaline and adrenaline secretion through the suppression of CRH secretion [116].

Rats that were fed diets containing extracts high in both falvanoid and total antioxidant activity for 6 weeks before being subjected to 48 hours of exposure to 100% normobaric O_2 showed no loss in striatal muscarinic or cerebellar GABAergic receptor sensitivity [118]. These oxygen-induced decreases in neuronal function have been shown to be sensitive to aging and have been associated with behavioral deficits [113].

Recent studies indicated preventive effects of garlic extracts for brain atrophy [119] as well as learning and memory impairments [120] in the senescence-accelerated mouse.

3.6. Vitamins

Chronically insufficient vitamin supply for vitamin C, thiamin, riboflavin, cobalamin, and folate causes many unfavorable psychometric changes [121]. Administration of folic acid, vitamin C, and to a lesser extent thiamin, as compared with placebo, in men with an initial suboptimal folate status, led to

a decreased emotional lability, increased activeness and concentration, higher extroversion and lower introversion, greater self-confidence, and a markedly improved mood [121]. In volunteers with an initial mild-to-moderate vitamin C deficiency, supplementation led to decreased nervousness, less depression, and increased emotional lability [121].

3.6.1. *Folic Acid*

Recent studies indicate the importance of folate in nervous system development. Folate is important in 1-carbon metabolism [122], contributing carbon atoms to purines, thymidine, and amino acids. In addition, methylation reactions involving folate may be important in the formation and maintenance of neuronal and glial membrane lipids [123]. Clinical trials indicate that folate supplementation (15 mg/day) for 6 months improve outcome in depressed and schizophrenic patients treated with standard pharmacotherapy [124]. Intake of folic acid has been linked to other psychiatric conditions as well as to deficits in learning and memory, particularly in the elderly [125]. The mechanism by which folate modifies mood is hypothesized to be related to its role in 1-carbon metabolism [122]. In the form of methylenetetrahydrofolate, the methyl donor in methionine synthesis from homocysteine, folate may help maintain adequate methionine pools for *S*-adenosylmethionine synthesis [126]. The link to mood involves the role of *S*-adenosylmethionine as a cofactor in methylation reactions in catecholamine synthesis and metabolism [127].

3.6.2. *Vitamin B_6*

The physiologically active forms of vitamin B_6 are enzymatic cofactors in many reactions of mammalian nitrogen metabolism, including the metabolism of most amino acids and neurotransmitters. Indeed, a dietary deficiency of vitamin B_6 affects tissue concentrations of amino acids and neurotransmitters in rats and humans [128, 129]. In general, vitamin B_6 deficiency leads to a decrease in the concentrations of most amino acids. Brain amino acids most commonly reported to be affected by deficiency are serotonin, dopamine, and γ-aminobutyric acid (GABA) [128, 129]. Studies indicated that excess dietary vitamin B_6 affects brain and serum concentrations of some amino acids and binding properties of cortical serotonin receptors in a biphasic pattern [130]. Also, large doses of vitamin B_6 can affect central nervous system function [130, 131] and neurotransmitter concentration [132]. Further, high concentrations of pyridoxal phosphate suppress activation of transcription, while vitamin deficiency enhances responsiveness to steroid hormone [133], decreased GABA, and increased central nervous system irritability [134]. Also, pyridoxine is essential in the conversion of

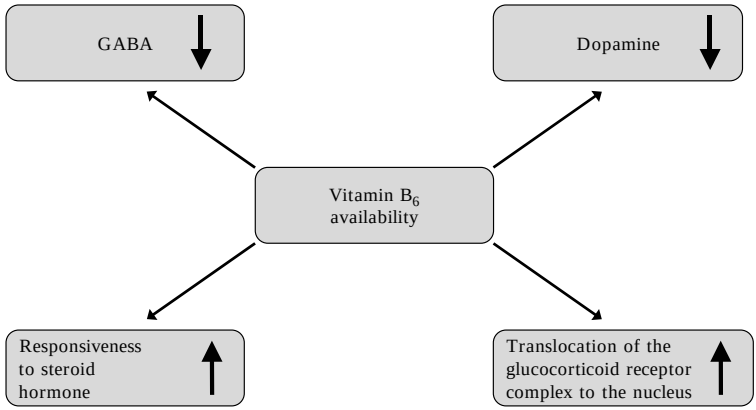

FIG. 2. Physiological effects of vitamin B_6 availability [135].

l-dihydroxyphenylalanine to dopamine. Side effects of excessive l-dihydroxyphenylalanine include dystonia and dyskinesia [134] (Fig. 2).

3.6.3. *Vitamin B_{12}*

Lack of cobalamin may lead to severe neurologic disorders, which have been described in strict vegetarians, especially in infants and toddlers [136, 137]. A wide variety of neurologic symptoms and signs have been described, such as ataxia, loss of cutaneous sensation, diminished or hyperactive reflexes, dementia, loss of memory, psychoses, and disturbances of mood [138, 139]. In contrast, complete or partial improvement, following vitamin B_{12} supplementation, has also been reported in memory loss [139], depression [140], and psychosis [141].

3.6.4. *Vitamin C*

Ascorbic acid affects the regulation of the levels of the circulating thyroid and adrenal cortical hormones [142]. Ascorbic acid is essential for optimal steroid hormone functions and this suggests an involvement of ascorbic acid in steroid synthesis mechanisms [143]. Vitamin C depletion led to a significant increase in plasma cortisol without an increase in ACTH [144]. In animal studies, ascorbic acid deficiency caused an increase in plasma cortisol concentration [145]. In rats, vitamin C pretreatment enhanced the release of endogenous GC such as to delay the turnover of the tracer cortisol in plasma [146]. In humans, there was a distinct increase of plasma cortisol about 2 hours after vitamin C application. This increase was concomitant with an increase in urinary 17-hydroxycorticosteroids [147].

3.6.5. *Vitamin E*

Vitamin E, as the major chain-breaking lipid-soluble antioxidant, would be expected to be important for functional integrity of all biological membranes. The neuropathological changes of vitamin E deficiency in humans are very similar to those in rats and rhesus monkey [148], and the resulting neurological syndrome is characterized by areflexia, peripheral neuropathy, cerebrallar involvement with gait and limbio ataxia, and decreased proprioception and vibration sense [149]. A defect in the fast anterograde and retrograde axonal transport has been reported in vitamin E-deficient rats [150]. Cerebellum seems to be active in the metabolic utilization of vitamin E. This could be the reason for cerebellar damage during experimental vitamin E deficiency and for the incidence of cerebellar symptoms in clinical vitamin E deficiency [151].

3.7. Minerals and Trace Elements

Certain minerals' balance is crucial for a healthy nervous system and neuronal susceptibility. Several reports suggested that the body electrolytes (sodium, potassium, calcium, and magnesium) and the level of some trace elements play an important role in stress susceptibility.

3.7.1. *Iodine*

Intrauterine iodine deficiency is well established as the cause of cretinism and lesser degrees of cognitive and motor disability [152]. There is a suggestion that low serum thyroxine (T_4), secondary to iodine deficiency, is linked to poor intellectual performance of the people residing in iodine-deficient areas [153]. Triiodothyronine seems to be the active hormone with respect to neurological development in the fetus and is synthesized in the brain from thyroxine transported from fetal plasma across the blood–brain barrier [154]. Despite good evidence that maternal thyroxine contributes substantially to fetal thyroxine in the later weeks of pregnancy [155], further maternal transfer is insufficient for fetal requirements if hypothyroidism is severe [156]. Hypothyroid patients show slowing of intellectual function and speech and have memory deficits [157]. Epidemiological studies indicate that school-aged children living in iodine-deficient villages were found to have poorer levels of IQ, cognitive, and motor function than school children in iodine-sufficient villages [158–163].

3.7.2. *Iron*

Iron is involved in numerous neurological functions. Iron deficiency is strongly related to the severity of anemia with a 50% decrease in muscle myoglobin content, cytochrome oxidase activity, and electron transport

capacity in skeletal muscle, concurrent with a 50% decreased oxygen transport capacity because of anemia [164]. The physiological manifestations of iron deficiency have also been noted in immune function, thermoregulatory performance, energy metabolism, and exercise or work performance [165, 166]. Restless legs syndrome has been described as being causally related to iron-deficiency anemia [167]. The brain obtains iron primarily via transferrin receptors expressed on endothelial cells on the brain microvasculature [168, 169]. The rate of iron uptake into the brain is increased when the iron status of the subject is low and is decreased when the iron status is higher [170]. Regions of the brain rich in iron, that is, the substantia nigra, globus pallidus, and nucleus accumbens, are far less affected by dietary iron deficiency than are other regions such as the cortex or the striatum that have less iron content [171]. Iron deficiency during lactation in the rat results in significant loss of regional brain iron that is distinct from those regions that lose iron with dietary restrictions later in life [172]. Restoration of brain iron with later aggressive dietary iron repletion also resulted in incomplete restoration of abnormalities in dopamine metabolism and in behaviors related to dopamine [172, 173].

3.7.3. *Magnesium*

Magnesium supplementation has been beneficial in a wide variety of conditions, such as neuropsychiatric disorders, ischemic heart disease and cardiac arrhythmias, asthma, diabetes, and chronic fatigue, in which magnesium deficiency has not always been substantiated [174, 175]. Extra- and intracellular magnesium levels have been shown to be genetically controlled in humans [176], and genetic differences in magnesium utilization may account for differences in vulnerability to magnesium deficiency and differences in body response to stress [177].

3.7.4. *Zinc*

Zinc is a trace mineral that is involved with RNA and DNA synthesis and is critical to cellular growth, differentiation, and metabolism [178, 179]. In the central nervous system, zinc is concentrated in the synaptic vesicles of specific glutaminergic neurons, which are found primarily in the forebrain and connect with other cerebral cortices and limbic structures. During synaptic events, zinc is released and passes into postsynaptic neurons, serving as a neurotransmitter [180]. Zinc deficiency may compromise behaviors necessary for cognitive functioning including activity and attention [178, 181]. In psychiatric patients, zinc deficiency may affect emotionality and response to stress [182, 183]. Studies of severe zinc deprivation in monkeys before weaning showed that zinc-deficient animals were emotionally less mature; this was demonstrated by their difficulty with separation and the increased

protective behavior by their mothers [184]. The association between zinc deficiency and an increased risk of anxiety and depression may be related to the stress reaction observed in zinc-deprived animals [185].

In the trials done on Chinese children and Mexican-American children from Texas, it was found that zinc-supplemented children demonstrated superior neuropsychological performance, particularly in reasoning, when compared with controls [186, 187]. In contrast, in a further study [188], Bangladeshi infants supplemented with zinc had slightly lower developmental scores, possibly because of micronutrient imbalance.

4. Meal Frequency

Timing of meal can influence the effects of meals on cognitive behavior. Early studies indicate that stunted and previously malnourished 9- and 10-year-old Jamaicans performed less well on tests of short-term memory and problem-solving ability when they had not eaten breakfast than when they had eaten a morning meal [189]. Undernourished children's performance on a test of verbal fluency was significantly better when they had consumed a school breakfast than when they had not [190]. Experimental evidence suggests that omitting breakfast negatively affects cognitive functioning [191].

5. Meal Size

The degree to which lunch moderates subsequent cognitive performance and mood may be mediated by meal size. In a study investigating the effects of meal size on attention and mood indicated that subjects who ate a larger than usual lunch made more errors on attention and search tasks than those who ate a normal-sized lunch, or one smaller than usual [192]. No differences in mood were noted as a function of meal size. Graig and Richardson [193] found that young men made significantly more errors on a letter-cancellation project after eating a large lunch but tended to make fewer errors after small lunch. Performance improved to a greater degree after the small lunch in subjects who typically ate a heavy lunch than in those who ate a light lunch. Afternoon snacks may also have positive effects on cognitive performance [194].

The effects of an evening meal on cognitive performance and mood indicated that subjects had stronger feeling and were more proficient and interested 1–3 hours after meal consumption than subjects who did not consume the meal [195]. Additionally, 90 min after the meal, the subjects who had eaten the meal completed more sentences on a logical-reasoning task than those who had not eaten.

6. Appetite

Appetitive behavior is complex and multifaceted. Stress reactivity, both physiological and psychological, may distinguish overeaters from undereaters [196]. There is much evidence from animal studies that HPA axis function can profoundly influence expression of appetite and regulation of body weight [6]. The recently discovered new gut peptide, ghrelin, an endogenous ligand for the growth hormone secretagogue receptor [197], seems to be involved in the control of food intake and energy balance. In fact, centrally injected ghrelin produces a sustained food intake in rodents, and ghrelin blood concentrations and mRNA expression in the stomach are increased by fasting and decreased by feeding [198]. Recent data have suggested a possible stimulatory effect of ghrelin on the HPA axis activity in experimental animals [199]. In humans, gherlin has a positive effect on glucose levels and negative effects on insulin concentrations [200]. Ghrelin concentrations are decreased in human obesity [201].

The stress-sensitive adrenal steroids modulate neurotransmitters which affect appetite, such as β-noradrenergic systems, neuropeptide Y (NPY), and galanin [29]. Exposure to stress increases NPY [202], which can increase appetite [203].

7. Emotion

Emotional arousal has been associated with both increased or decreased food intake and weight [204, 205]. Self-reported increases in negative mood (mood reactivity) during the stressor were also significantly positively related to caloric consumption, whereas mood reactivity on the control day was not related to consumption that day [33]. Cortisol reactivity and mood are two somewhat independent indices of stress reactivity and found that both were related to eating after stress, but not after rest [33]. Psychophysiological response to stress influences subsequent eating behavior [33]. It is possible that women more vulnerable to stress, in their mood responses and cortisol reactivity, may be at particular risk of stress-induced eating and weight gain [33].

In humans, McCann *et al.* [206] examined the effects of variation in workload on food intake and serum lipids with a small group of female office workers. The workers reported a higher energy intake and a higher percentage of energy as fat in two high-workload periods compared with the normal work period. Higher energy intake was reported from a study of dietary habits associated with exam time among university students [207]. The dietary data support modest increases in energy, fat, and sugar intake in

periods of high work stress compared with periods of low work stress [206, 208]. Either prolonged or frequent work stress could result in increasing the likelihood of weight gain and increased cardiovascular risk [208]. Also, behavioral factors as one of the psychobiological mechanisms can affect health [208]. Individual variability in dietary responses to stress in relation to levels of dietary restraint has been identified in a number of experimental studies [209–212]. Restrained eaters did not just eat more overall, they specifically ate more sweet and fatty foods in the high-work-stress session, and the hyperphagic response was greater among those who had a larger increase in perceived stress between the low- and high-workload sessions, implicating emotional reactions in the response [208].

8. Metabolic Acidosis

Chronic metabolic acidosis (CMA) is a frequent acid-base disturbance generated by extrarenal loss of base (e.g., diarrhea), increased acid production (e.g., organic acidosis such as ketoacidosis), or impaired renal acid excretion (i.e., renal failure and inherited or acquired forms of renal tubular acidosis). The modern Western-type diet in humans, which is rich in animal protein, has been implicated as a cause of lifelong mild CMA with secondary bone catabolism caused by the induction by this diet of an obligatory daily acid load (endogenous acid production) due largely to endogenous oxidation of cationic and sulfur-containing amino acids [9]. Plasma bicarbonate concentration decreases progressively when endogenous acid production is increased by menu changes among normal foodstuffs in normal subjects [213]. CMA has also been shown to cause a significant increase in corticosteroid excretion [214, 215]. In a small human study, experimental-induced acidosis was also associated with an increase in cortisol excretion [216] however, another similar study in humans did not show any increase in cortisol secretion, although plasma aldosterone levels significantly increased [217]. Also, hyper-GC response to CMA has been demonstrated in humans [214] and rats [216]. GCs lead to a dramatic decrease in bone mineral density, either when endogenously in excess or when administered exogenously [218]. The mechanism by which GCs decrease bone density is multifactorial. The osteopenia appears due to a complex combination of direct effects on bone formation [219–222] and resorption [219, 220, 223], and indirect effects on calcium homeostasis, including decreased intestinal calcium absorption [223].

Animal and human studies suggest that metabolic acidosis stimulates an increase in cortisol production [214–216]. CMA can increase cortisol production and both acidosis and cortisol induce osteopenia. In muscle, acidosis is

known to stimulate protein and essential amino acid breakdown through the ubiquitin–proteasome proteolytic pathway, a mechanism that requires GCs [214]. A very mild Western diet-induced CMA (a degree of acidosis that would not be recognized by applying diagnostic acid-base criteria found in textbooks) results in a state of increased cortisol secretion and plasma concentration and provides several novel findings in humans regarding the possible causality of the Western diet in the etiology of osteoporosis [224]. Ingestion of neutralizing alkali per se, as exchanged for chloride in the absence of other experimental maneuvers (e.g., concomitant potassium supplement), can result in urinary calcium retention and suppression of biochemical markers of bone resorption [224].

GCs decrease bone formation via suppression of osteoblast maturation and promotion of apoptosis [222]. They inhibit production of osteoprotegerin, a soluble neutralizing receptor produced by osteoblasts, which limits osteoclastogenesis [225]. GC-induced osteopenia *in vivo* has been well characterized [222, 226], although the exact mechanism of induction of the resultant loss of bone mineral is not entirely understood. In general, there appears to be an uncoupling of bone remodeling to favor bone resorption over bone formation [223]. The net osteopenia observed *in vivo* after GC treatment is probably due to a complex combination of direct effects on bone formation and resorption as well as indirect effects to inhibit intestinal calcium absorption and increase renal calcium excretion [220, 223, 227].

Ingestion of an ordinary acidogenic Western diet to normal young adult subjects results in a mild CMA in association with a state of increased cortisol secretion and plasma concentration, altered divalent ion metabolism, and increased bone-resorptive indices [224]. In humans, CMA results in hypersecretion of cortisol [216]. Previous studies reported a nonsignificant, 77% increase in urinary cortisol excretion in acid-fed compared with nonacid-fed human subjects [228].

9. Glucocorticoids

GCs are also known to affect development [229, 230], memory [231, 232], fear and anxiety [233], and the immune system [234–236]. For example, acute elevations of GCs enhance immune responses in rats to provide important immunoprotective effects [237]. Many studies find a positive correlation between plasma GCs and the expression of feeding behavior. Food intake is normally highest at the time of day when baseline GCs show a peak, and intensity of feeding can be shifted with GC treatment [11]. Food intake may be mediated through GC-induced stimulation of NPY [13, 238–240], and catecholamines [13], or through inhibition of the anorexigenic peptide CRF [241, 242].

GC levels above the seasonal baseline are generally correlated with protein utilization and muscle tissue breakdown [243, 244]. Experimental manipulations confirm the involvement of elevated GC levels in such metabolic changes: GC administration induces protein loss and muscle atrophy in a variety of species [245–248]. GCs may also promote gluconeogenesis through enhanced substrate delivery. Studies in dogs suggest that cortisol treatment enhances *de novo* glucose synthesis through increased amino acid uptake in the liver [249, 250]. Studies in a variety of vertebrate species verify the role of GCs in the acute provisioning of glucose. Treatment with GC equivalents increases plasma glucose in birds [251, 252] and can reverse hypoglycemic effects of insulin injections [253]. It is important to note that GC manipulations may not always affect plasma glucose levels [254, 255].

GCs support a heightened physiological state by promoting availability of lipid energy from adipose tissue stores [11, 234]. For example, cortisone administration significantly reduces the stored triglyceride fraction of adipose tissue in lizards [256]. Also, GCs increase lipogenesis and fat deposition in the liver [252, 256, 257]. GCs may contribute to this process of FA oxidation by making available amino acids for use as citric acid cycle intermediates [258]. Further, GCs may promote fat mobilization by inhibiting glucose uptake in adipose tissue [234].

In relation to health, dysfunction of the HPA axis has been implicated in particular in dysphoric disorders, such as major depression [259], whereas hypercortisolemia in Cushing's syndrome is accompanied by physical symptoms, such as accumulation of abdominal adipose tissue, together with muscular atrophy of the limbs, providing powerful evidence of the well-established metabolic and nutritional consequences of chronic hypercortisolemia [94]. Normal physiological variations in cortisol level in humans have a significant direct influence on macronutrient metabolism [25, 26]. Also, the ability of cortisol to increase plasma FFA levels may underlie the emerging link between cortisol and abdominal obesity, together with its associated metabolic syndrome [27].

In healthy males, exogenous GC administration increased daily food intake compared with placebo [260]. Cushing's patients, with elevated cortisol levels selected high-fat foods twice as often as normal weight subjects and three times as often as overweight controls [261]. Laboratory test indicated that among healthy women, high cortisol reactors ate significantly more food following a cognitive stress task compared with low cortisol reactors [33]. In an earlier study [262], subjects undergoing a stressful task before a meal showed increased plasma cortisol during the task, which was then followed by a suppressed cortisol response to the meal. Cortisol clearly plays an important role in energy regulation, increasing available energy through gluconeogenesis and lipolysis. Adrenalectomy and GC receptor

antagonists prevent or reverse obesity [263], whereas administering corticosterone leads to increased appetite for sucrose [264], hyperphagia, and weight gain [265]. In cancer patients, prednisolone significantly increased appetite, compared with a control group [266]. In healthy men, administering cortisol for 4 days led to slightly increased energy expenditure but dramatically increased food intake [260].

Approximately 90–95% of plasma cortisol is bound to CBG, albumin, and erythrocyte membranes [267], whereas only the free fraction is thought to be physiologically active. Cortisol in saliva is a valid measure of free cortisol levels and is easily sampled repeatedly without distress [8, 267].

10. Monoamine

Serotonin (5-HT) is involved in the regulation of the HPA axis in mammals [268, 269]. In several studies, the ratio of 5-hydroxyindoleacetic acid (the major 5-HT metabolite) to 5-HT brain concentrations has been found to correlate with plasma levels of cortisol, suggesting that the action of brain 5-HT on the HPA axis is stimulatory [270, 271]. An increased activity of serotonergic neurons in the brain is an established consequence of stress, and a decline activity of these serotonergic neurons has been demonstrated in disturbances of mood and depressive disorders [272]. Increased brain 5-HT activity appears to be a prerequisite for maintaining control over cognitive information processes [273] and is involved in learning and memory [274]. As brain 5-HT secretion increases under stress [275, 276], chronic stress may result in frequently elevated concentrations of cerebral 5-HT. As 5-HT function increases under acute stress, brain 5-HT concentrations may be exhausted under continuous stress exposure. As a consequence, the serotonergic system of subjects prone to stress (high-stress-vulnerable subjects) may become more sensitive to dietary-induced alterations in L-tryptophan availability because of compensatory receptor sensitization [277, 278]. Depletion of the precursor of 5-HT synthesis, tryptophan, has been found to increase depressive mood in healthy subjects and subjects with a prior history of depressions [279].

5-HT is synthesized from the essential amino acid L-tryptophan and the first and rate-limiting step in the biosynthesis of 5-HT is the hydroxylation of L-tryptophan to 5-hydroxytryptophan. Since the enzyme tryptophanhydroxylase, catalyzing the hydroxylation of L-tryptophan, does not seem to be saturated by L-tryptophan *in vivo*, the rate of this reaction appears to be restricted by L-tryptophan availability in mammals [280]. Elevated dietary intake of L-tryptophan has been reported to result in increased brain levels of L-tryptophan and elevated rates of 5-HT synthesis and metabolism [281, 282].

The carrier transporting L-tryptophan across the blood–brain barrier is nonspecific, also transporting other large neutral amino acids (LNAAs; i.e., tyrosine, phenylalanine, leucine, isoleucine, and valine). Brain levels of L-tryptophan will thus not only depend on plasma levels of L-tryptophan, but also on plasma levels of other LNAA competing for the same carrier [280, 281]. A CHO-rich, protein-poor diet increases the ratio of plasma tryptophan to the sum of the other LNAAs, giving tryptophan an advantage in the competition for access into the brain [34, 283–285]. Markus *et al.* [286] found that a CHO-rich, protein-poor food diminished the depressive mood and cortisol response to controllable as well as uncontrollable laboratory-induced stress in highly stress-prone human subjects. Acute stress elevates brain L-tryptophan concentrations [287], an effect that appears to be mediated by a stress-induced elevation of sympathetic activity and circulating plasma catecholamines [288]. An activation of the sympathetic system stimulates lipolysis, resulting in elevated plasma levels of nonesterified FAs, competing with L-tryptophan for binding to albumin and thus elevating the plasma pool of free L-tryptophan available for uptake into the brain [268]. Sympathetic activation may also increase brain L-tryptophan uptake by affecting the permeability of the blood–brain barrier [268].

Tyrosine is the precursor to the catecholamine neurotransmitters dopamine, norepinephrine, and epinephrine. Analogous to the ability of tryptophan to stimulate serotonin production, elevating tyrosine concentrations in brain catecholamine neurons (particularly dopamine and norepinephrine neurons) can stimulate transmitter production. This effect occurs in actively firing neurons but not in catecholamine neurons that are quiescent or firing slowly [289, 290]. Tyrosine administration to depressed patients improved their mood, but although catecholamine production was enhanced, the treatment did not elevate mood [291]. Growdon *et al.* [292] showed that tyrosine elevated dopamine production in the central nervous system of patients with Parkinson's disease, a serious, debilitating disorder, the cause of which is thought to involve a loss of dopamine neurons and which is typically treated by administering the immediate dopamine precursor, L-dopa. Further, tyrosine administration appears to improve cognition and performance in soldiers under stressful conditions [293, 294].

11. Stress

The key hormonal pathway that governs the endocrine response to stress is the HPA axis. Elevation of serum corticosterone, the endpoint of stress-induced activation of the HPA system, is frequently used as a stress indicator, and a convincing number of studies have found several measures indicative

of a hyperactive HPA axis in depressed patients [295]. Compared with controls, women with anorexia nervosa [296], bulimia nervosa [297], binge eating disorder [298], and the night eating syndrome [299] had higher basal cortisol. Exaggerated cortisol responses to stress have been observed in women with anorexia nervosa [300], bulimia nervosa [301], and obesity [302]. Eating is thought to be suppressed during stress because of anorectic effects of CRH, and increased during recovery from stress because of appetite-stimulating effects of residual cortisol [33, 303]. Cortisol secretion is a major component of the stress response [301], and it has been implicated as a potential mediator for increased energy intake in healthy males [260] and females [33].

Typically, responses to stress result in anorexia and, if the stress is sufficiently persistent, weight loss. The longstanding view is that stress produces sympathetic arousal that results in reductions rather than increases in eating. For example, in rats, both a single social defeat stressor [304] and a 2- hour immobilization stressor [305] resulted in a significant reduction of food intake and body weight. Overeating has been observed in rats following a stress, following a period of caloric restriction [306] only in those given highly palatable food [307]. In humans, dieters are more likely to report stress hyperphagia compared with nondieters who are more likely to report stress hypophagia [308].

The chronic mild stress procedure in rats decreased sucrose intake per unit body weight, while sucrose intake in a nonstressed control group did not change [309].There was not any correlation between body weight and sucrose intake. Sucrose intake was reduced while body weight remained unaffected [309]. The largest effect was obtained after 2 weeks of the stress protocol, this effect was attenuated afterward [309]. D'Aquila *et al.* [310] also observed a recovery of "reward behavior" in the chronic mild stress model. Overeating of "comfort foods" in humans may be stimulated by cortisol in response to stress, which can result in abdominal obesity [311].

Although acute elevation of cortisol plays a protective role during stress, persistently elevated levels promote insulin resistance and abdominal obesity [312, 313]. Insulin resistance might be followed by both dyslipidemia and elevated blood pressure [314]. Cortisol secretion as a response to perceived stress is a powerful factor regulating disease-generating events in the periphery. This seems to be particularly the case when the HPA axis functions with low reactivity and poor feedback control [315]. Most studies have observed that chronic stress overactivates the HPA axis and fuels insulin release, in turn activating abdominal fat storage [316].

Stress has been implicated as a primary trigger of overeating [317]. Delay in gastrointestinal transit time (an indirect measure of gastric emptying and intestinal motility) has been observed in lean participants, but not the obese, after exposure to both active and passive coping tasks [318].

Self-reported retrospective [319, 320] and prospective data [206, 208, 321] suggest that food choice does change under stress, with a tendency toward a relative increase in sugary, fatty (often snack-type) foods. Grunberg and Straub [32] extended the usual laboratory paradigm by providing participants with a range of foods differing in taste qualities (sweet, salty, and bland), although these were still snack foods presented incidentally to the main task of viewing a film (used for stress induction). In the stressed group men ate less than men in the control group. In women there were no significant differences, although stressed women did show a trend toward a modest increase in consumption of sweet and bland foods with no change in intake of salty foods. These gender differences may have reflected differences in dietary restraint, which is higher in women [322–324].

Women and restrained eaters consume more calories and fat under stress [206, 208] and shift their food choices away from meal-type foods, such as meat and vegetables, toward snack-type foods [308]. In contrast, men and unrestrained eaters show either little difference or a reduction in food intake under stress [32, 325]. Stress did increase intake of sweet fatty foods in emotional eaters. In addition, women scored more highly on emotional eating than men [320]. There is evidence that snack consumption may be more susceptible to stress than meals [308, 326]. An alternative neurohormonal mechanism for stress-induced preferential selection of sweet fatty foods is suggested by evidence that such highly palatable foods can themselves relieve stress through release of endogenous opioids [327, 328]. Stress changed people's perception of saccharin's bitterness and sweetness, as it does in rats [329], but the direction of change depended on aspects of temperament such as trait arousability, pleasure, and dominance.

Psychological stress seems to increase oxidative stress [330]. Early studies indicate that psychological stress decreases DNA repair [331] and inhibit radiation-induced apoptosis [333] in human blood cells. This may mean that oxidative damage may persist during psychological stress and may increase the likelihood of a pathological development [330]. In contrast to the tendency of chronic stress to elevate baseline cortisol, it appears to decrease testosterone, both in animals and in humans [334, 332].

12. Conclusion

Modification of the diet and changes in frequency cause changes in endocrine and monoamine synthesis. These changes may affect the susceptibility to stress. In general, foods of plant origin have to be preferred. Reduction and modification of dietary fat may be helpful. In particular, a diet rich in MFAs and n-3 FAs is advisable. Further, a large part of the diet has to be of

complex CHOs. These modifications can produce consistent changes in cortisol and its binding globulin. Cortisol reactivity and mood are two somewhat independent indices of stress reactivity, and it was found that both were related to eating after stress. Further dietary modification may affect the availability of L-tryptophan. L-tryptophan has been reported to result in increased brain levels of L-tryptophan and elevated rates of 5-HT synthesis and metabolism. Increased brain 5-HT activity appears to be a prerequisite for maintaining control over cognitive information processes and involvement in learning and memory. A decline activity of these serotonergic neurons has been demonstrated in disturbances of mood and depressive disorders. Not only modification of the diet but also timing of meal can influence the effects of meals on cognitive behavior. Also, omitting breakfast negatively affects cognitive functioning. Further, the effects of meal size on attention and mood indicated that subjects who ate a larger than usual lunch made more errors on attention and search tasks than those who ate a normal-sized lunch or one smaller than usual. Performance improved to a greater degree after the small lunch in subjects who typically ate a heavy lunch than in those who ate a light lunch. Further, afternoon snacks may also have positive effects on cognitive performance.

For positive influences of stress, mood, and cognitive function plant foods might be preferred. They are rich in phytochemicals and vitamins, which show a variety of positive effects on health. Also, consumption of green and yellow vegetables everyday may lower the incidence of some stress syndromes (e.g., irritation and sleeplessness).

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MENOPAUSE, ESTROGEN, AND GONADOTROPINS IN ALZHEIMER'S DISEASE

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1. Abstract

For decades, Alzheimer's disease (AD) has been linked to aging, gender, and menopause. Not surprisingly, this led most investigators to focus on the role of estrogen. While undoubtedly important, estrogen is unlikely the key determinant of disease pathogenesis. Rather, it appears that estrogen may

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work in conjunction with a novel determinant of disease pathogenesis, namely gonadotropins. The fact that gonadotropins, specifically luteinizing hormone, play a pivotal role in disease is apparent from significant etiological, epidemiological, and pathological evidences. Moreover, targeting gonadotropins appears to have beneficial actions as a therapeutic regimen.

2. Introduction

Dementia, a syndrome of many causes, defined as an acquired deterioration in cognitive abilities that impairs the successful performance of activities of daily living, is the result of disorders of cerebral neuronal circuits and is a consequence of the total quantity of neuronal loss as well as the specific location of such loss [1]. The most common cognitive ability lost with dementia is memory. In western countries, AD is the commonest cause of dementia, followed by vascular dementia, Parkinson's disease, and dementia due to alcoholism and drug/medication intoxication. Increasing age, however, appears to be the single strongest risk factor for dementia. Disabling memory loss increases with each decade above 50 years and, at autopsy, is most often associated with microscopic changes of AD. Today, whether or not dementia is an inevitable consequence of normal human aging remains a topic of controversy. Nevertheless, while some centenarians retain intact memory function with no evidence of clinically significant dementia, and while AD can occur at any age, we know that significant memory loss is seen in $\sim$10% of all persons above 70 years and AD is the causal factor in more than half of them. While a subtle cumulative decline in episodic memory, also termed as "benign forgetfulness of the elderly," is a natural part of normal aging, a significant portion of individuals with a mild cognitive problem, also referred to as mild cognitive impairment, that interferes with daily activities will progress to frank dementia, usually caused by AD. Depending on the cause of dementia, anatomically specific patterns of neuronal degeneration occur which dictate the clinical symptomatology. AD first involves the entorhinal cortex, spreads to the hippocampus, and eventually causes a relatively diffuse cortical degeneration [2]. It primarily presents with memory impairment and progresses to language deficits, with aphasia or other disturbance of language, and visuospatial deficits. If made after careful evaluation, a clinical diagnosis of AD is confirmed at autopsy 90% of the time [1]. Even though there is, at present, no definitive treatment for AD, it is important to detect. A diagnosis of AD would allow the treating physician to discontinue all unnecessary medication as well as identify and treat new intercurrent illness, find alternate ways to obtain history at ensuing visits, ensure the patient's medication is taken correctly, and help the patient and patient's family to deal with the disease.

Cognitive impairment results in a diminished quality of life and reduces the capability of the individual to live independently. This poses a serious public health problem. The increasing life expectancy of our population inevitably carries with it a rising incidence of cognitive symptomatology and age-related illnesses. The current number of 4 million individuals affected with AD in the United States today, if unchecked, is expected to rise to an estimated 14 million by the year 2050 [3].

Our incomplete understanding of the molecular mechanisms involved in cognitive decline and the pathogenesis of AD has impeded the development of effective therapy. Despite a large number of studies aimed at forestalling the onset and progression of AD, current therapeutic management studies have been, at best, limited to palliative treatment. It is crucial that we develop strategies to prevent, halt, or even reverse, decline in cognitive function in order to improve the quality of life of our aging population and avert a rise in health care costs. To achieve this goal, the molecular mechanisms involved in cognitive decline must be deciphered and better understood. Only then can successful diagnostic tools and therapeutic strategies for neurodegenerative diseases such as AD be devised. Here we review current knowledge, clinical and experimental, of the role of a changing hormonal environment during menopause and andropause in the pathogenesis of AD and, inevitably, the new direction in therapeutic options to which this understanding points.

In patients with AD, there is a progressive decline in episodic, working, and spatial memories [4, 5] which refer to the conscious recollection of facts and events. There is diminished capacity to form large-scale representations of the environment and difficulty in learning and orienting to unfamiliar settings [6, 7]. The mechanisms responsible for this type of behavioral decline have yet to be delineated. What has been described is the associated selective neuronal degeneration affecting the hippocampus and, to a lesser extent, other cortical brain regions. The hippocampus, a highly plastic area of the brain, is crucial in the modulation of the types of memory and cognition affected in AD. Importantly, the hippocampus is also one of the most age-sensitive areas in the brain. It is thought that greatly diminished plastic capabilities of this region lead to impairments in cognitive output.

3. Hypothesized Pathogenic Mechanisms of AD

The early appearance of histopathologic lesions in the hippocampus has been hypothesized to be one of the mechanisms that lead to cognitive dysfunction in AD. However, the etiologic events that lead to the two well-characterized histopathologic lesions of the disease, namely neurofibrillary tangles composed of hyperphosphorylated tau protein and senile plaques

composed of amyloid-β (Aβ) protein, are incompletely understood. Nonetheless, of the several hypotheses put forward to explain the pathogenesis of AD, the amyloid hypothesis has gained most attention. This is due to the finding that early onset AD results from mutations in either the amyloid-β protein precursor (AβPP) or the presenilins 1 and 2, proteins involved in the processing of Aβ [8]. However, studies using animal models do not support this theory. In transgenic rodent models that overexpress mutant AβPP, despite large depositions of Aβ in the brain, there is little or no neuronal loss [9]. In fact, there is increasing evidence to support the theory that Aβ deposition is a consequence rather than a causative factor in AD pathogenesis [10–13]. Other theories being considered as plausible explanations for the hallmark brain lesions seen in AD are tau phosphorylation [14–16], oxidative stress [17], metal ion dysregulation [18], and inflammation [19]. Tau, a microtubule-associated protein, serves to assemble and stabilize the microtubule. Tau hyperphosphorylation impairs its capacity to bind to microtubules and prevents the microtubules from functioning normally. Accumulating evidence supports the concept that oxidative stress generated by various mechanisms may be a major factor that contributes to the initiation and promotion of neurodegeneration [17, 20, 21]. Transition metals, such as iron, copper, aluminum, and zinc, have been found in high concentrations in the brains of patients with AD and studies suggest their involvement in the etiopathology of the brain changes seen in AD [22, 23]. Overall, these studies indicate that the environmental conditions in AD, exacerbated by imbalances in several metals, have the potential to catalyze and stimulate free radical formation and enhance neuronal degeneration. Other associated findings in AD include decreased cortical levels of acetylcholine, the enzyme choline acetyltransferase, nicotinic cholinergic receptors, and reduced norepinephrine in brainstem nuclei [2]. Each of these mechanisms, while they may have a role in the disease process in AD, cannot, when considered alone, sufficiently explain the spectrum of abnormalities found in AD [24]. The search therefore continues for a causative factor that would be present at the time of onset of the disease, predating the clinical features as we know them today, and which would serve as an initiator of the pathologic process and evolution of the disease.

4. Gender Dichotomy in AD: Role of Sex Steroids

Investigation of the epidemiological trends unique to AD yields conflicting data [25, 26]. The fact remains, however, that there is a higher prevalence [27–30] and incidence [31] of AD in aging women. There is also a higher incidence of Aβ plaques in brains of women as compared with men [32]. This gender-based disease predisposition is specific to AD and is not found in other degenerative diseases, such as Parkinson's disease, where men have a

higher prevalence and progression of disease. This has, naturally, focused attention on the roles played by diminishing levels during aging and reproductive senescence of estrogen and, to a lesser extent, testosterone, in the pathogenesis of AD.

The part played by sex steroids, and estrogen in particular, in age-related cognitive decline and AD has gained prominence due to a number of lines of evidence suggesting that postmenopausal estrogen deficiency may contribute to both the benign cognitive decline [33–35] and the etiology of AD in women [36, 37]. These findings are supported by epidemiological and observational studies indicating that hormone replacement therapy (HRT) lessens the risk of AD in postmenopausal women [38–42]. It is interesting, however, that reports suggest HRT to be protective only when administered during a "critical period," that constitutes the climacteric years. HRT is almost completely ineffective when given later during the postmenopausal years, during the latent preclinical stage of AD, the disease itself occurring much later in life [43–47].

The observed gender differences, in addition to the reported capacity of HRT to reduce AD risk in postmenopausal women, have led researchers to investigate a possible role for estrogen in the pathogenesis of AD. It is already known that estrogen has a proven ability to act as a neuroprotective agent by lowering the brain levels of Aβ [48], ameliorating the nerve cell injury caused by Aβ [49], and promoting synaptic plasticity and growth of nerve processes [50–52]. Additionally, estrogen is capable of reducing oxidative stress, increasing cerebral blood flow, and enhancing cholinergic function and glucose transport into the brain [53]. These effects are all known to exert a positive impact on the amelioration and prevention of AD. Several studies have been carried out on the effects of adrenal steroids on hippocampal plasticity [54–56]. One study in embryonic rat hippocampal cells suggests that a metabolite of dehydroepiandrosterone (DHEA) may be responsible for some of the functions ascribed to estrogens [57].

In men, although cessation of gonadal hormone secretion is not abrupt during midlife, levels of testosterone decrease gradually with aging. There is some evidence to show that higher levels of estradiol, arising through aromatization of testosterone, in elderly men may protect against some decline in memory during normal aging [58, 59]. Some authors go so far as to suggest that testosterone replacement therapy may alleviate memory loss in elderly men [60, 61].

Despite the large body of evidence supporting a role for estrogen in preventing age-related cognitive decline as well as AD, there have been recent contradictory studies [62]. While reduced estrogen levels have been reported in patients with AD as compared to controls [39], HRT using estrogen and progestin resulted in little improvement in cognitive function [40]. One must also consider the fact that a "critical period" in HRT-based protection against AD suggests that falling levels of steroid hormones that accompany

menopause/andropause cannot sufficiently account for patterns of AD susceptibility. Such inconsistent results merely reflect our incomplete understanding of the basic mechanisms in AD. They do, however, raise questions regarding an authentic role of sex steroids in the disease process. Add to this the fact that steroid hormone changes do not account for the observation that, in Down's syndrome, where serum levels of estrogen and testosterone are comparable to those in the general population, males are at a significantly higher risk for developing precocious AD-type pathology and cognitive alterations, and at an earlier age, than females [63]. This reversal in Down's syndrome of the normally female gender-based predisposition to AD cannot therefore be explained on the basis of lowered sex steroid levels.

The Women's Health Initiative (WHI) Memory Study, which reported negative cognitive effects following HRT with conjugated equine estrogen at an AD-vulnerable age, has raised the most recent and significant challenge to estrogen's protective role in AD etiology. The WHI study [46, 64], which demonstrated that HRT in postmenopausal women does not improve cognitive performance and may actually increase the risk of developing AD, has raised new questions concerning the role of sex steroid hormones in age-related cognitive decline, concurrent neuronal dysfunction, and the development of AD [46, 65–67]. Many hypotheses have been postulated to justify the results of the WHI study. Some aspects related to the form (estradiol vs conjugated equine estrogen) and the route of administration (oral vs transdermal) of estrogen, the choice of progestin (natural vs synthetic progestins), the high doses administered, and the type of treatment regimen (continuous vs cyclic) might be deserving of consideration (reviewed in [68–70]). The investigators also suggest that the negative results may be linked to the increased risk of stroke reported in the steroid treatment group. The relationship between microinfarcts in the brain and susceptibility to AD is most likely to be related but has not yet been well characterized [71].

It would seem, therefore, that even in the face of evidence supporting the protective role of estrogen on cognition and AD, the current controversy clearly reveals that falling levels of steroid hormones that accompany menopause/andropause cannot sufficiently explain patterns of age-related cognitive decline and AD susceptibility.

5. A Gonadotropin Evidence-Based Hypothesis for AD: High Gonadotropin vs Low Estrogen

In light of some findings challenging the protective role of estrogens in AD, as detailed above, it is our view that the evidence, so far, points to the probability that the differential effects of HRT may be, at least partially,

dependent on secondary hormonal changes in gonadotropins such as luteinizing hormone (LH). In support of this hypothesis, the levels of gonadotropins including LH are highest during perimenopause and early menopause [72], when HRT has been observed to be most successful in preventing dementia [73, 74].

Each hormone of the hypothalamic-pituitary-gonadal (HPG) axis, including hypothalamic gonadotropin releasing hormone (GnRH), LH, follicle stimulating hormone (FSH), estrogen, progesterone, testosterone, activin, inhibin, and follistatin, is involved in regulating reproductive function by participating in a complex feedback loop. Hypothalamic secretion of GnRH initiates the feedback process by stimulating the anterior pituitary to secrete the gonadotropins, LH and FSH. These gonadotropins then bind to receptors on the gonads and stimulate oogenesis/spermatogenesis as well as the production of the sex steroids. Sex steroids complete the negative feedback loop by decreasing GnRH and gonadotropin secretion from the hypothalamus and pituitary gland (reviewed in [75]).

The balance of this feedback loop can shift during menopause (and ''andropause'') and result in an increase in the production of gonadotropins such as LH and FSH. In postmenopausal women, changes mediated by decreased action of estrogen [76] result in large increases in the concentration of serum LH and FSH [77]. Likewise, men (but to a lesser degree than women) also experience an increase in these hormones as their reproductive function declines during andropause [78]. Surprisingly, despite these well-documented hormonal changes, the effects of loss of negative feedback and consequent increased circulating gonadotropins on the aging brain remain largely unexplored. This may be because these hormones are traditionally considered to be involved in peripheral nervous system and not CNS function.

Evidence suggests, however, that gonadotropins such as LH also have CNS function. It is recognized that significant elevations of LH are found in vulnerable neuronal populations in AD as compared to controls [76]. LH, like estrogen, is capable of modulating cognitive behavior [79]. Luteinizing hormone receptors (LHR) are present in the brain [80–82] and, like estrogen receptors, are highly expressed in the hippocampus [80], which, as detailed above, is an age-sensitive, highly plastic area of the brain, crucial in the modulation of the types of cognition affected by aging and severely deteriorated in AD. LH itself has been found in the cytoplasm of pyramidal neurons of normal subjects, but in increased concentrations in AD brain compared to control [83]. This is likely due to the fact that LH and FSH are present in the cerebrospinal fluid of postmenopausal women (with LH higher than FSH in its cerebrospinal fluid to serum ratio) [84] as well as the fact that LH is known to cross the blood-brain barrier [79], again suggesting effects outside the reproductive system [85]. A study attempting to elucidate the downstream

consequences of binding of brain LH to its receptors showed increased expression of steroidogenic acute regulator protein, which regulates the first step in steroidogenesis, in vulnerable neurons and other cell types in AD brains as compared to controls [86]. Of particular importance was the finding that steroidogenic acute regulator expression colocalized with LHR expression. The authors suggest that LH binding to LHR in AD susceptible neurons induces potentially pathogenic signaling events in the brain and that the LH-regulated steroidogenic pathways may play a role in AD. LH represents the only factor, thus far, that explains the gender predisposition in the incidence of AD as well as its reversal in Down's syndrome [87]. In the normal population, where women have a higher prevalence of AD, LH is higher in women but in Down's syndrome, where serum LH is higher in men despite, sex steroid levels being comparable to the general population, men are at a higher risk of developing AD-type changes [88]. LH, unlike other hormones of the HPG axis, is highest during perimenopause and early menopause [72] when HRT has been observed to be most successful in preventing dementia [73].

As a consequence of these observations, there is growing evidence supporting a role for gonadotropins, particularly LH, in AD pathogenesis [89]. A twofold increase in circulating gonadotropins has been reported in patients with AD compared to age-matched controls [90, 91]. Of paramount importance in elucidating disease pathogenesis is the understanding of regional and temporal selectivity of neuronal death in AD. Lei *et al.*[80] have shown that regional expression of LH receptors in the brain corresponds to regional vulnerability seen in AD with the highest neuronal density being in the hippocampus. We have found correspondingly significantly elevated LH in vulnerable neuronal regions in AD as compared to controls [83]. Such increases appear to be a very early change, serving to predict neuronal populations at risk of degeneration and death. Elevations in LH parallel the ectopic expression of cell cycle and oxidative markers that represent one of the initiating pathologic changes preceding neuronal degeneration by decades [92, 93]. The highest concentrations of the hormone, human chorionic gonadotropin, which is homologous to LH, also correspond to the time of most rapid cell proliferation (i.e., the fetal period). This leads us to suspect that neuronal elevations in LH could play a major role in the mitogenic abnormalities documented in AD [83].

While AD is not traditionally characterized as a disease of cell division, it has been postulated that the unscheduled initiation of a mitotic division cycle in a mature, normally postmitotic neuron leads to abortive reactivation of a variety of cell cycle components and ultimately to the demise of the cells in AD [94–96]. The involvement of cell cycle-related events in the etiology of AD is supported by neuronal changes such as the ectopic expression of numerous markers of the cell cycle [97], organelle kinesis [98], and cytoskeletal alterations including tau phosphorylation [99]. Reactivation of the mitotic

signaling pathways by extracellular receptor kinase (ERK) and by mitogen-activated protein kinase (MAPK) [16, 100, 101], which are coincidentally also known to be upregulated by gonadotropins, including LH [102], lend even more compelling support to this theory. Furthermore, such mitotic alterations are not only one of the earliest neuronal abnormalities in the disease [97, 101, 103], but also would lead to all of the other pathological changes reported in the disease [94]. LH is a powerful mitogen [102], and given the temporal and spatial overlap with mitotic changes in AD [83] (unpublished observations), it is likely that elevations in LH are responsible for inappropriate cell cycle reentry in neurons [83, 94]. Admittedly, this does not preclude the involvement of the other hormones of the HPG axis that also exhibit significant changes in serum concentrations later in life.

In testing the hypothesis that LH plays a role in cognition and AD, we have shown in experimental studies that, while LH did not alter AβPP expression in a neuroblastoma cell line, LH did alter AβPP processing toward the amyloidogenic pathway *in vitro* by increased secretion and insolubility of Aβ, decreased secretion of AβPP, and increased levels of AβPP-C99 [104]. In the same study, treatment with leuprolide acetate, a selective GnRH agonist that has been shown to markedly reduce LH by downregulating the pituitary GnRH receptors [104, 105], led to 3.5- and 1.5-fold reduction in total brain Aβ 1–40 and 1–42 concentrations, respectively, in C57B1/6J mice [104]. We have recently found that by experimentally abolishing LH using leuprolide acetate in an animal model of AD, the AβPP transgenic mouse improved cognitive performance and decreased Aβ deposition [106]. Additionally, overexpression of LH in a transgenic mouse model leads to cognitive deficits and neuronal function/plasticity changes, the effects of which are believed to be receptor specific [107]. These findings, together with data indicating that LH modulates AβPP processing *in vivo* and *in vitro* [104] toward amyloidogenic pathways, indicate that LH may play a crucial and direct role in age- and AD-related cognitive and associated neuronal function decline. Even more compelling evidence that LH may be a key player in AD is seen in the results of a recently completed phase II clinical trial (http://clinicaltrials.gov/ct/show/nct00076440?orden=6) which show evidence of stabilization in cognitive impairment and activities of daily living in female AD patients treated with high doses of leuprolide acetate (http://www.secinfo.com/d14D5a.z6483.htp, pp. 56–64).

6. Conclusion

Several reports in the literature support a protective role for estrogen on cognition and preventing AD. Results of animal and human studies, however, indicate that the "window of opportunity" for HRT is at the time of

menopause or immediately after ovariectomy. HRT administration following a considerable delay after menopause or ovariectomy has little beneficial effect on cognition. As a consequence of these observations, and the fact that LH concentrations are significantly higher in AD patients than in age-matched controls, there is growing evidence supporting a role for gonadotropins, particularly LH, in AD pathogenesis. The regional and temporal selectivity of neuronal cell death in AD, the regional expression of LH receptors, and the LH concentration in vulnerable brain areas lend credence to this theory. Gender dichotomy in AD prevalence, as well as gender reversal in Down's syndrome, makes it reasonable to believe that the observed beneficial effects of HRT are mediated, at least in part, through LH suppression. The most compelling evidence, however, is the response to high dose leuprolide acetate, a selective GnRH agonist, seen in female AD patients who show stabilization of cognitive impairment [108, 109]. In order to fine-tune this therapy, the intricate molecular mechanisms involved in producing this response, the effect on neuronal mediators, and structural changes in vulnerable brain regions have to be pursued.

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IMMUNOSUPPRESSION ROUTED VIA THE KYNURENINE PATHWAY: A BIOCHEMICAL AND PATHOPHYSIOLOGIC APPROACH

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Abbreviations

APCs	antigen-presenting cells
CpG	cytidyl guanosyl
CTLA-4	cytolytic T-lymphocyte antigen-4
DCs	dendritic cells
GAS	interferon-γ activating site
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IRF	interferon regulatory factor
ISRE	interferon-stimulated response element
LPS	lipopolysaccharide
NAD	nicotinamide adenine dinucleotide
NK	natural killer
NO	nitric oxide
TDO	Trp 2,3-dioxygenase

1. Abstract

In the past years, it has been shown that kynurenines pathway is a regulator of both the innate and the adaptive immune responses. Particularly, the initial enzyme of this pathway, indoleamine 2,3-dioxygenase (IDO), is implicated in maintaining tolerance during pregnancy, and also can be expressed in tumors to avoid the immune attack. In this chapter, we will describe how the kynurenine pathway affects the immune system with important implications both in physiology and in pathology. The incorrect activation or blockade suppressive properties of the kynurenine pathway are also implicated in a number of other diseases such as AIDS or autoimmune diseases.

2. Introduction

Most of the research effort in the immune system has been oriented to discover the mechanisms that activate the cellular and the humoral responses. However, it is beyond any doubt that there are also peripheral mechanisms that maintain tolerance and participate in health and disease. For example, self-reactive T cells that escape clonal deletion in the thymus must be suppressed by peripheral tolerance to avoid autoimmune diseases. Also, during evolution, a mechanism of maternal tolerance has been developed to avoid fetal rejection. Different mechanisms that contribute to peripheral tolerance have been described [1], and seem to act in an overlapping fashion. The kynurenines pathway has recently joined this selected group [2], and its importance is progressively being unraveled.

Tryptophan (Trp) is the rarest of the 20 amino acids found in proteins, and is an essential amino acid in animals. Apart from being one of the bricks that constitute proteins, it is the precursor of important molecules such as serotonin, melatonin, and nicotinamide adenine dinucleotide (NAD). Kynurenine was initially isolated by Kotake in 1926 and Trp degradation products through the route initiated by IDO are collectively known as kynurenines. The kynurenine pathway was initially situated in the context of the immune system as a part of the antitumoral [3] and intracellular antimicrobial [4] defense machinery. More recently, the kynurenine pathway has been shown to be a regulator of both the innate and the adaptive immune responses.

Although there is controversy related to the mechanism involved in suppression, and even in the type of immune cells that express IDO activity, it is fairly clear that the kynurenine pathway plays a pivotal role in regulating the balance between activation and inhibition of the immune system. In this chapter, we will describe how the kynurenine pathway affects the immune system with important implications both in physiology and in pathology.

3. Trp-Kynurenine Degradation Pathway

Trp metabolism through the kynurenine pathway is summarized in Fig. 1. Kotane discovered the enzyme that catalyzed the conversion of Trp to *N*-formylkynurenine and named this enzyme Trp pyrrolase, renamed after as Trp 2,3-dioxygenase (TDO, EC 1.13.11.11) [5]. This is one of the two first enzymes that initiate Trp degradation through the kynurenine pathway; the other is IDO (EC 1.13.11.17) [6, 7]. These enzymes are the rate-limiting enzymes of the Trp degradation pathway and are tightly regulated. Both enzymes catalyze the oxidative cleavage of the indole ring of Trp

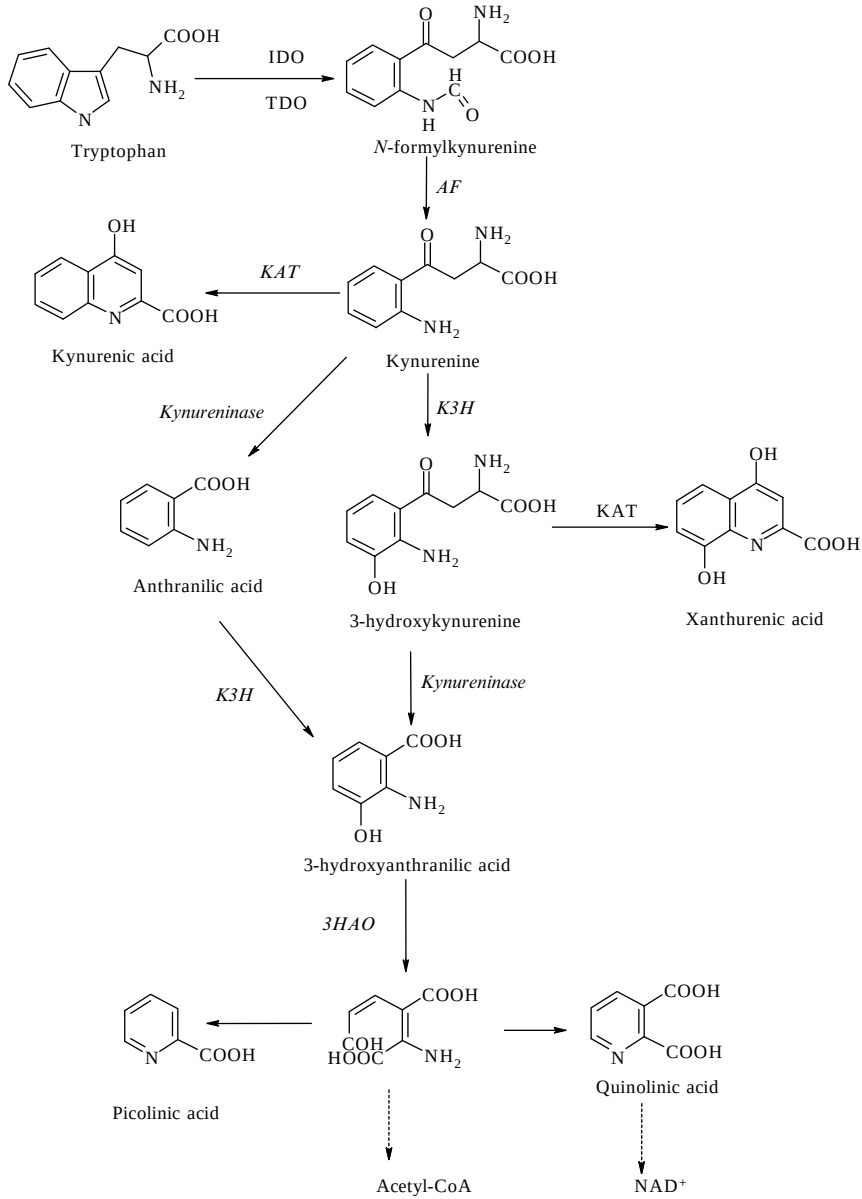

FIG. 1. Scheme of the kynurenine metabolic pathway in mammalian cells, in which indoleamine 2,3-dioxygenase (IDO) catalyzes the initial and rate-limiting step. Most of the Trp not used for protein synthesis is metabolized through this pathway. IDO: indoleamine 2,3-dioxygenase; TDO: tryptophan 2,3-dioxygenase; AF: arylformamidase; K3H: kynurenine 3-hydroxylase; KAT: kynurenine aminotransferase; 3HAO: 3-hydroxyanthranilic acid oxidase.

rendering *N*-formylkynurenine, which loses the formyl group to form the more stable product kynurenine. This reaction occurs spontaneously in acidic conditions or catalyzed by arylformamidase (EC 3.5.1.9). The aromatic ring of kynurenine is hydroxylated by the flavin-dependent kynurenine 3-monooxygenase (EC 1.14.13.9) yielding 3-hydroxykynurenine. Both kynurenine and 3-hydroxykynurenine undergo the conversion to anthranilic acid or 3-hydroxyanthranilic acid by a reaction catalyzed by kynureninase (EC 3.7.1.3). Alternatively, kynurenine and 3-hydroxykynurenine can be desaminated by the vitamin B6-dependent enzyme kynurenine aminotransferase (EC 2.6.1.7), rendering kynurenic acid or xanthurenic acid, respectively. 3-Hydroxyanthranilic acid can be further metabolized by 3-hydroxyanthranilic acid oxidase (EC 1.13.11.6) to the unstable intermediate aminocarboxymuconic semialdehyde, which is rapidly metabolized to either quinolinic acid or picolinic acid, or totally oxidized to CO_2 and H_2O. Quinolinic acid is a precursor of NAD^+ that links this degradation pathway with the intracellular redox status.

3.1. Comparison Between IDO and TDO

TDO and IDO are the enzymes that generate kynurenine from Trp in humans. These two enzymes notably differ in terms of location, structure, substrate specificity, and regulation [8]. TDO is a constitutive hepatic enzyme consisting of a homotetramer of 167 kDa subunits noncovalently bounded [9]. It shows substrate specificity for L-Trp, which binds with low affinity and uses O_2 in the oxidative ring cleavage. TDO activity is induced by Trp; other inducers are kynurenine, hystidine, tyrosine, and phenylalanine. It has been observed that the enzymatic activity increases after a protein-rich meal and is maintained several hours afterwards [10]. In addition, the gene has a glucocorticoid response element [11].

IDO is a 45-kDa monomeric enzyme with high affinity for Trp, though other indoles such as 5-hydroxy-Trp and serotonin can also serve as substrates. In addition to O_2, superoxide anion can be a source of oxygen in the oxidative reaction.

3.2. Distribution of the Kynurenine Pathway Enzymes

TDO is a hepatic enzyme, while IDO has a wider tissue distribution, with high activity in lungs, small intestine, and placenta, and lower activity in stomach and spleen [12]. In extrahepatic tissues, Trp is mainly metabolized to kynurenine by IDO. Not all the enzymes of the kynurenine pathway are simultaneously expressed in tissues, but hepatocytes are the only cells containing all the enzymes of the kynurenine pathway leading to complete

oxidation of Trp and also to NAD^+ synthesis [10, 13]. It has been shown that enzymes of the kynurenine pathway are differently expressed in tissues depending on the species [14]. In human astrocytes and neurons, there is a low activity of kynurenine 3-hydroxylase, kynureninase, and kynurenine 3,4-dioxygenase, and in lungs there is no detectable activity of kynurenine 3-hydroxylase [15]. Because of the absence of these enzymes, the production of the neurotoxic quinolinic acid is very limited in the brain in normal situations [15], but can be produced during inflammation [16]. In the immune system, monocytes have high kynurenine 3-hydroxylase, kynureninase, and 3-hydroxyanthranilic acid oxidase activities [15]. Once induced by IFN-γ, these cells also have high IDO activity, degrading Trp to quinolinic acid and producing NAD^+[17].

NAD^+ is an essential cofactor in many cellular reactions, and crucial for DNA repair and maintaining the redox balance. Significant amounts of NAD^+ are formed through the TDO-mediated kynurenine catabolic route in the liver [10, 13], but other cells such as astrocytes and neurons depend on extracellular sources of this cofactor [15]. IFN-γ-stimulated macrophages can produce NAD^+, protecting themselves against free radical production [17]. In diets with normal supply of niacin, this route is secondary for the synthesis of NAD^+ and $NADP^+$, but Trp becomes important as a precursor of NAD^+ in niacin-deficient diets. In chronic insufficient supply of Trp and niacin, a vitamin-deficient disease, pellagra, can occur.

4. Biochemistry of IDO

4.1. Molecular Biology of the Indo Gene

The Indo gene is situated in the short arm of chromosome 8, and consists of 10 exons that span 15 kbp [18]. There are no reports on alternative splicing or other posttranscriptional modifications. The human Indo cDNA encodes a single protein of 403 amino acids and 42 kDa [19], which presents 61% homology with murine IDO [20]. This protein has high hydrophobic amino acid content and 5% of carbohydrates [21].

The Indo gene has been conserved during the past 600 million years of evolution related with the Trp metabolism, and in other species has even evolved to perform different functions such as the oxygen transport in IDO-like myoglobin protein found in some gastropod mollusks [22]. Immunoregulation of T-cell immunity was not the initial role of such an ancient enzymatic activity, although it might have been recruited later in evolution for such purposes. We can therefore place this gene in the category of innate

immunity based on its appearance before the evolutive merge of the adaptive immunity [23]. During adaptation, Indo gene could have served as a bridge between innate and adaptative immunity, participating in the antiparasitic and antibacterial defense developed by macrophages, and in the modulation of T cells by dendritic cells (DCs).

4.2. Catalytic Properties of IDO

IDO is a heme-monooxygenase very different from other human heme-monooxygenases such as cytochrome P-450 [24]. The X-ray crystal structure of human IDO shows two α-helical domains with the heme group between them. The catalytic mechanism consists of the binding of O_2 to the ferrous heme, and the binding of L-Trp that enables the interaction between the –NH group of indole and the proximal atom of dioxygen producing *N*-formyl kynurenine [24]. In the absence of a reducing environment, Fe^{2+} is easily oxidized to Fe^{3+} by inactivating the enzyme. Dihydroflavin mononucleotide or tetrahydrobiopteridine has been proposed as a cofactor that maintains IDO-iron in a reduced state *in vivo* [25].

IDO has a wide substrate specificity, and catalyzes the oxidation of L- and D-Trp; however, high concentrations of L-Trp inhibit the activity of the enzyme [26]. 1-Methyl-Trp is a Trp analog that competitively inhibits IDO [27], but not TDO. 1-Methyl-Trp inhibits transport system L [28], therefore blocking the Trp entry into the cell and affecting protein synthesis and probably also affecting immune functions. In addition, it has been shown that 1-methyl-Trp can modulate DCs function independently of its action on IDO [29]. This compound is frequently used in the IDO enzyme research.

4.3. Posttranslational Regulation of the IDO Activity

IDO is highly induced during maturation of monocyte-derived DCs [30, 31], but full IDO activation requires further stimulation, mainly by IFN-γ, which implies a posttranslational regulation by some type of IDO activator [32]. For example, even though mouse splenic DCs express IDO protein at comparable levels, only the CD8α^+ subset has IDO activity after exposure to IFN-γ [33]. Orabona *et al.* [34] showed that IFN-γ induces in $CD8^+$ DCs the expression of the transcription factor IFN regulatory factor (IRF)-8, which induces IDO expression and downregulates the membrane protein adapter DAP12. The downregulation of DAP12 is necessary for full IDO activity, which implies that the loss of DAP12 expression could be a hallmark of the IDO-competent DCs.

Another mechanism of regulation of the IDO function is through modification of either the apoprotein or the cofactor. Nitric oxide (NO) is an IDO functional modifier acting at these two levels, in general, decreasing IDO activity during inflammation [35]. Also, some antioxidants such as pyrrolidine dithiocarbamate, a NF-κB inhibitor, decrease IDO activity post-translationally [36]. Another mechanism of regulation of the IDO activity is through the supply of the prosthetic group heme to the apoenzyme: the inhibitor of the heme biosynthesis, succinylacetone, can also inhibit IDO activity without affecting protein content [36].

5. IDO Induction

5.1. Induction of IDO by IFNs

IDO is induced by cytokines, mainly IFN-γ, in many tissues [3, 37]. In general, the effect of IFN-γ inducing IDO expression is more pronounced than the one provoked by IFN-α/β, and even the capacity of IFN-γ to induce IDO activity has been used in bioassays to measure this cytokine activity [38]. Professional antigen-presenting cells (APCs) such as monocytes, macrophages, and DCs, express IDO following IFN-γ exposure [37, 39, 40], and the maturation of monocytes to macrophages increases the capacity of IFN-γ to induce IDO [41]. Also, CD4+ can express IDO upon stimulation with IFN-α [42] and IFN-γ [43]. As IFN-γ is the most potent IDO inducer, the mechanism will be discussed in detail in the following paragraphs.

5.2. Induction of IDO by Cytolytic T-Lymphocyte-Associated Antigen 4-Immunoglobulin Fusion Protein

CD80 (B7–1) and CD86 (B7–2) are surface molecules present on DCs that interact with two counterreceptors expressed by lymphocytes (CD28 or CTLA-4). Cytolytic T-lymphocyte antigen-4 (CTLA-4) is a negative regulator of T-cell activation transiently expressed on T cells after activation that can bind CD80 and CD86 on DCs. The binding of the CTLA-4-Fc fusion protein to CD80 induces IDO expression in DCs [44, 45], and also in CD4+ T cells [46]. The mechanism of IDO induction in DC is indirect; CTLA-4-Fc induces IFN-γthat, in an autocrine or paracrine fashion, leads to IDO expression, Trp depletion, and conceivably immunosuppression. DCs obtained from mice deficient in either IFN-γ or signal transducer and activator of transcription 1 (STAT1) do not produce kynurenine after CTLA-4-Fc treatment *in vitro* [44]. In addition to the soluble form (CTLA-4-Fc), also CTLA-4 anchored in the membrane of regulatory CD4+ can induce IDO expression in DCs [47].

5.3. Induction of IDO by Unmethylated Cytidyl Guanosyl Oligodeoxynucleotides

The toll-like receptors (TLR) are membrane proteins that bind to pathogen-expressed molecules. The receptor, TLR9, binds cytidyl guanosyl (CpG) oligodeoxynucleotides present in the genome of bacteria and viruses, but not in human genome. This receptor is expressed by B lymphocytes and plasmacytoid DCs; and after TLR9 activation, there is an induction of IFN-α, IFN-γ, and other proinflammatory cytokines initiating the innate and adaptive immune responses [48]. Also, bacterial DNA and its synthetic immunostimulatory oligodeoxynucleotide analogs induce IDO, which is one of the defense mechanisms against intracellular pathogens [49]. However, the binding of bacterial DNA to TRL9 in lungs produces intense IDO expression, provoking TH1 and TH2 suppression [50].

The activation of TH1 cells by synthetic CpG-rich oligodeoxynucleotides has been shown to have therapeutic activity in mouse models of cancer, and infectious and asthma/allergy diseases; and for this reason, several clinical trials have been initiated using CpG-rich oligodeoxynucleotides [48]. However, repeated systemic administration of CpG-rich oligodeoxynucleotides causes lymphoid follicle destruction and immunosuppression [51] that could be related, at least in part, to the kynurenine pathway activation. For this reason, applications using CpG-oligodeoxynucleotides as adjuvants in vaccines for human use should consider this possible immunosuppression [52] and evaluate kynurenine production.

The effect of CpG oligodeoxynucleotides on IDO expression is related to the route of inoculation, which affects the cell type first encounter and the type of microenvironment [52]. Systemic administration of CpG oligodeoxynucleotides could induce IDO expression, provoking an activation of the kynurenine pathway and leading to T-cell suppression [53, 54]. However, subcutaneous application of antigen plus CpG oligodeoxynucleotides enhances antigen-specific T-cell activation in local lymph nodes [54].

5.4. Induction of IDO by 4-1BB

4-1BB (CD137) is a member of the tumor necrosis factor (TNF) receptor superfamily expressed on the surface of activated T cells and other leukocytes including natural killer (NK) cells and mature DCs [55]. 4-1BB-mediated costimulation greatly enhances T-cell-mediated immune responses helping in eradicating stabilized tumors and virus clearance [56, 57]. But the same 4-1BB costimulation produces opposite results, inducing tolerance and improving autoimmune diseases [55]. In a mouse model of rheumatoid arthritis, the administration of an anti-4-1BB agonist antibody induces

IFN-γ-mediated IDO expression in macrophages and DCs [58]. This enzyme leads to Trp depletion and produces kynurenines causing depletion of antigen-specific CD4+ cells. This prevents the development of collagen type II-induced arthritis in the mouse model. Interestingly, *in vivo* administration of the IDO inhibitor 1-methyl-Trp completely abolishes the therapeutic effect of the agonist anti-4-1BB monoclonal antibody. Similarly, the anti-4-1BB antibody improves experimental autoimmune uveoretinitis by IDO-dependent mechanisms [59].

5.5. Signaling Pathways Involved in IDO Induction

The Indo gene promoter has several response elements that confer responsiveness to IFN-α/β, and more potently to IFN-γ. In the promoter region of the Indo gene, there are two very important sequence elements, interferon-stimulated response element (ISRE) found in IFN-α/β-inducible genes, and interferon-γ activating site (GAS) found in IFN-γ-inducible genes, and there is also an MHC-II X,Y box-like motif [18, 60]. The presence of these elements confers responsiveness to inflammatory cytokines, mainly to IFN. Both IFN-α/β and IFN-γ induce IDO in monocytes, but IFN-γ is the most potent because the ISRE region is less stimulated by IFN-α/β [60]. Upon binding to its receptor, IFN initiates the Jak-STAT signaling pathway that activates specific DNA sequences in the cell nucleus (Fig. 2). STAT1 can bind directly to the GAS element and indirectly, through the IRF-1, to the ISRE element. There is a cooperative action between IRF-1 and STAT1 to mediate IDO induction upon stimulation with IFN-γ [61]; and, for example, mice deficient in IRF-1 or IFN-γ cannot induce IDO during infection [62].

There are also IFN-γ-independent mechanisms to induce IDO and not mediated through the STAT1 or IRF-1 pathway. Lipopolysaccharide (LPS) stimulates IDO expression and acts synergically with other cytokines such as TNF-α, in a mechanism involving NF-κB and/or the activity of the p38 mitogen-activated protein kinase[63].

6. Measurement of Kynurenines in Human Biological Fluids

There are several commercially available antibodies to detect IDO protein; however, this enzyme is posttranslationally regulated, so the immunological detection does not necessarily imply activity [64], and the enzymatic activity should be measured when studying functionality. IDO activity is easily inactivated *in vitro* by oxygen, which oxidizes Fe^{2+} of the heme group to Fe^{3+}. To measure IDO activity in cellular extracts, it is necessary to reduce the Fe^{3+}, usually with methylene blue plus ascorbic acid [26]. Catalase is also

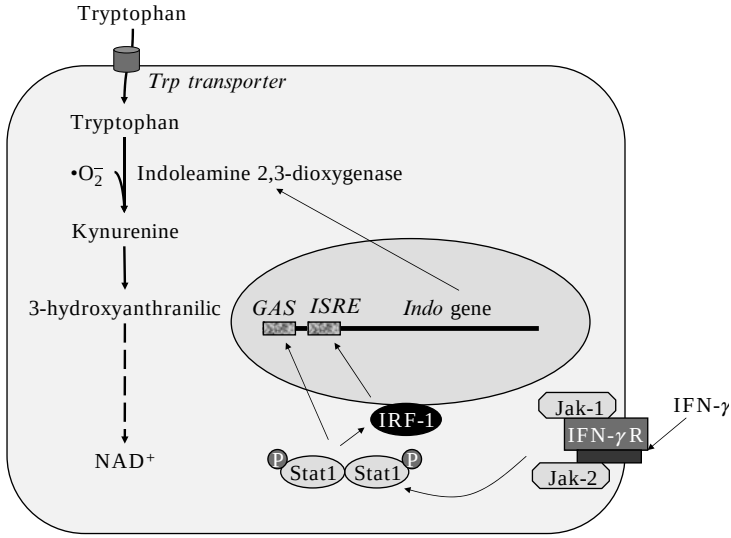

FIG. 2. IFN-γ induces indoleamine 2,3-dioxygenase (IDO) gene (Indo) transcription through JAK-STAT1 signaling pathway, and participating also the interferon regulatory factor-1 (IRF-1). In the Indo promoter, there are two interferon-stimulated response elements (ISRE) and a single IFN-γ activating site (GAS) implicated in the response to IFN-γ. IDO catabolizes Trp in an oxidative reaction that consumes oxygen radicals. To be metabolized by the cytoplasmic enzyme, Trp should enter into the cell though a membrane transporter.

added to eliminate the hydrogen peroxide produced by the reducing system. In these conditions, Trp is readily converted to formylkynurenine by IDO enzyme (Fig. 1). Boiling the reaction product for several minutes eliminates the formyl group rendering kynurenine. Then, Trp and kynurenine are usually simultaneously measured by high-performance liquid chromatography using reversed phase C18 columns [65]. Trp is detected by its natural fluorescence at an excitation wavelength of 285 nm and an emission wavelength of 365 nm, and kynurenine is measured using an ultraviolet detector set at a wavelength of 360 nm. Some authors have measured kynurenine by a colorimetric method using the Ehrlich reagent; however, it is very unspecific and prone to react with many compounds that interfere and mislead the quantitation [66]. There are physiological variations in the serum concentrations: Trp and kynurenine concentrations are 15% higher in men than in women [65], and intense exercise increases serum levels of kynurenine [67]. An indirect form to estimate IDO activity is quantifying the kynurenine/Trp ratio in biological fluids [59].

Quinolinic acid is a neurotoxic kynurenine metabolite involved in several neurodegenerative disorders [68]. For this reason, different methods have

been developed to quantify this compound in brain tissue and cerebrospinal fluid (CSF), usually by gas chromatography-mass spectrometry [69, 70]; and there is a regional variation of concentration in the brain [71]. Specific antibodies have been used in immunohistochemistry to detect cells capable to synthesize quinolinic acid. Probably due to the different sensitivity of both methods, in normal brain tissue quinolinic acid can be quantified, but it is not immunologically detected [68]. In inflammatory neurological diseases, where the concentration of quinolinic acid can rise several hundred times, this compound can be detected by immunohistochemistry in tissues [68].

Kynurenines are mainly eliminated by renal excretion [72, 73]. Thus, alterations in kidney function cause retention of kynurenines. Indeed, a marked elevation of kynurenine and quinolinic acid in serum and CSF in renal failure patients has been observed [70, 74]. This elevation causes an accumulation of kynurenines in different tissues, probably playing a role in the pathogenesis of the uremic syndrome [75]. It can be postulated that these compounds might be involved in the relative immunosuppression that affects patients suffering from chronic kidney disease.

7. Nitric Oxide and the Kynurenine Pathway

NO is generated from l-Arg by the enzyme nitric oxide synthase (NOS). There are three NOS isoenzymes, two of them (eNOS and nNOS) are constitutive and produce low quantities of NO and the third form is inducible (iNOS) and produces large quantities of NO. This compound has important immunomodulatory properties [76]. In enzymes carrying the heme group, NO can bind to this prosthetic group modifying the catalytic activity, either activating [77] or inhibiting [78], depending on the enzyme. Alternatively, NO can form peroxynitrite that reacts with tyrosine residues producing nitrotyrosines, which change the protein properties [79]. As IDO is a heme-protein, NO can bind its heme prosthetic group, inhibiting IDO enzyme activity [80–82]. Similarly, extensive IDO protein nitration due to elevated peroxynitrites output, as produced by iNOS, inhibits IDO activity [35, 82, 83]. This inhibition affects the entire kynurenine pathway as IFN-γ-induced synthesis of NAD^+ by macrophages is markedly increased in the presence of an NOS inhibitor [17]. NO favors IDO degradation by the proteasome [84], which could be related to IDO nitration because nitration of tyrosine residues in proteins induces accelerated degradation of modified proteins by the proteasome [85].

However, low micromolar concentrations of NO, as produced by eNOS, stimulate IDO activity, even when there is IDO protein nitration [35]. Coexpression of eNOS and IDO is found in human monocytes [86, 87] and in placenta [2]. As a result, eNOS can participate in the immune tolerance in an IDO-dependent fashion. However, when there is an inflammatory attack,

in which iNOS is induced as a part of the immune response, the high NO production deactivates the immunosuppressive IDO enzyme, thus favoring a more intense immune response [35].

NO interferes with the tolerogenic effect of kynurenines in the immune system, as demonstrated in a murine model of type 1 diabetes mellitus. Certainly, the high expression of peroxynitrites in DCs in predisposed nonobese diabetic (NOD) mice provokes the inactivation of STAT1 in these cells [88]. The blockade of this signaling route impairs the IFN-γ-mediated Trp degrading pathway, which interferes with the tolerance to self-antigens [88]. Restoration of this blockade allows IDO expression in DCs and the development of tolerance.

Kynurenine metabolites also modulate iNOS expression, but while picolinic acid can increase [89], 3-hydroxyanthranilic acid can inhibit IFN-γ-dependent iNOS mRNA expression and activity in macrophages [90].

8. Role of IDO in the Defense Against Infectious Pathogens

One of the most simple and ancient host defense against pathogens is the depletion of nutrients, such as iron-chelating proteins. The kynurenine pathway, by depleting Trp, can locally impair the growth of microbes. Soon after the discovery of IDO, it was observed that this enzyme is notably induced in mouse lung after an intraperitoneal administration of bacterial LPS [91], leading to Trp degradation and increased plasma kynurenine levels [92]. IFN-γ inhibits IDO-dependent growth of group B streptococci [93], intracellular pathogens (e.g., *Chlamydia psittaci*, *Leishmania donovani*, and *Toxoplasma gondii*) [94, 97], and viruses (e.g., *cytomegalovirus* or Herpes Simplex Virus) [95, 96]. This effect is mediated by increased Trp degradation, and inhibition could be reversed by the addition of Trp excess. Infection with *Candida albicans* produces an IFN-γ-dependent IDO induction in DCs and polymorphonuclear neutrophils that inhibits fungi growth, and in this case the antioxidant properties of IDO and kynurenines have a central role [98].

TNF-α can increase the IFN-γ-dependent IDO induction, potentiating the antiviral action of this cytokine [96]. IDO expression induced by IFN-γ in airway epithelial-like cells provokes exaggerated IL-6 and IL-8 responses to TNF-α and LPS challenge [99]. This could help to explain why respiratory viral infections can increase inflammatory responses to concurrent or secondary bacterial challenges, thereby worsening respiratory distress [99].

9. IDO as an Immunosuppressive Molecule

As it has been mentioned before, earlier literature considered the Trp degrading pathway a mechanism to inhibit pathogens growth and tumor proliferation. This point of view was challenged in 1998 when Munn and

Mellor [2] demonstrated that Trp degradation protected mice fetuses from maternal rejection. Soon later, it was shown that cells express IDO to limit T-cell proliferation in response to antigens: transfection of Indo gene into tumor cell lines confers them the capacity to inhibit T-cell responses [100]. Since then, a considerable amount of evidence has been accumulated regarding the immunosuppressive role of the kynurenine pathway and its role in developing tolerance (summarized in Fig. 3).

9.1. IDO-Mediated Suppression by Innate Immunity

Macrophages are the first defense line against pathogens, and the binding of bacterial molecules to receptors on the surface macrophages triggers the generation of the respiratory burst and phagocytosis. However, macrophages can also downregulate T-cell response, which is, at least in part, mediated by the kynurenine pathway. Monocytes differentiated to macrophages with macrophage colony stimulating factor (M-CSF) and suppress the proliferation of CD3-stimulated T cells [101]. To reach this suppressive state, macrophages should be induced to synthesize IDO, mainly by IFN-γ. T cells induce IDO in macrophages by an IFN-γ-dependent mechanism, which implies that these cells should be immunocompetent and previously activated. The order of cytokine treatment in culture is crucial, because when monocytes are previously treated with IFN-γ they do not achieve this T-cell

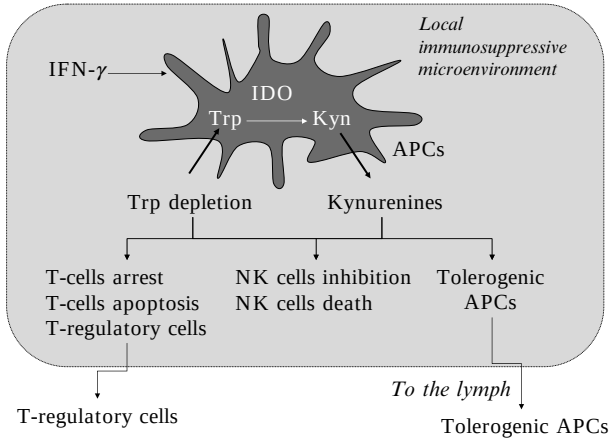

FIG. 3. Immunosuppressive effect of the kynurenine pathway. IFN-γ can induce the kynurenine pathway in antigen-presenting cells (APCs) producing immunosuppression in the microenvironment, and can also induce suppression at distance by migration of tolerogenic APCs to lymphatic nodes or by inducing regulatory T cells.

suppressive function once differentiated with M-CSF [102]. T-cell arrest is mediated exclusively by IDO induction without participation of any other macrophage inhibitory mechanism since addition of purified IDO protein also impairs the proliferation of phytohemagglutinin-stimulated T cells [103]. Stimulated T cells can initiate a response entering cell cycle, but they are halted at the middle of G1 when there is IDO activity in the microenvironment [101, 102]. This arrested situation of T cells cannot be overridden by Trp restoration only, but a second round of stimulation together with Trp addition is also necessary [101].

NK cells are another tool of the innate defense that can also be inhibited by the kynurenine pathway. Trp depletion can downregulate Ag-specific T-cell clonal expansion leading to suppression in response. Also, kynurenine inhibits phytohemagglutinin-stimulated NK cells proliferation [103], probably through cytotoxic mechanisms [104]. However, the NK-cell response, which does not need a clonal expansion, is downregulated by a direct effect on the NK-cell killing capability [105]. In this case, kynurenines seem to exert an efficient suppressive effect; and effectively, kynurenine can inhibit NK-cell responses induced by IL-2 and IL-12 [105]. Suppression is achieved at concentrations and for periods of time that do not cause cytotoxicity, but impair the upregulation of specific activatory surface receptors that bind triggering ligands on target cells. Indeed, L-kynurenine prevents the IL-2-induced upregulation of the activating cellular receptors NKp46 and NKG2D, responsible for NK-cell triggering, and consequently decreases killing capacity of NK cells [105]. The suppressive effect of kynurenine is transient as NK cells regain the phenotype and function after the metabolite removal. A similar effect on NKp46 is observed when L-kynurenine is present during IL-12 stimulation of NK cells.

9.2. IDO-Mediated Suppression by Adaptive Immunity

Different murine models have demonstrated that the functionally active kynurenine pathway suppresses T-cell-mediated immune responses. DCs are the most important APCs, specialized to induce or to suppress T-cell responses [106]. As it occurs with other cell types, IFN-γ induces functional IDO enzyme in DCs—an effect that is enhanced by CD40 ligand and LPS [107]. Of special interest is the demonstration by Grohmann *et al.* that CTLA-4-Ig upregulates IDO in murine DCs via an IFN-γ-dependent mechanism [44]. Plasmacytoid DCs do not express IDO and are not tolerogenic under basal conditions; there are multiple ligands and cytokines that induce the expression of IDO giving a tolerogenic phenotype to plasmacytoid DCs in mice [108]. CD28-Ig also induces IDO in plasmacytoid DCs in SOCS3-deficient mice through IFN-γ-induction and the IFN-like actions of IL-6

[108]. More recently, it was reported that CD200R ligation by the soluble CD200-Ig fusion protein induces IDO in plasmacytoid DCs through an IFN-α/β-elicited signaling pathway [109].

Maturation of human monocyte-derived DCs with TNF-α plus PGE2 induces IDO expression [30, 110]. Munn *et al.* [40] have shown that a population of nonadherent human DCs, characterized by CD123 and CCR6 expression, produces IDO protein, but the enzyme is only active after stimulation with IFN-γ. However, other authors have challenged these findings [111], and although this discordance has been attributed to the methodology used to differentiate DCs in culture [112], to detect IDO function and protein, and to analyze DCs function of T-cell stimulation [112, 113], the controversy is maintained [113]. Furthermore, DCs comprise a heterogeneous population, in which the differentiation and maturation culture conditions are very important in order to attain IDO-mediated suppression, as observed in the case of macrophages [102]. Moreover, not all the types of DCs have IDO activity [40, 111, 112]. For example, when maturation is induced with CD40 ligand, DCs express inactive IDO, which is even downregulated by IFN-γ. On the contrary, the addition of IL-10 together with CD40 ligand and IFN-γ generates mature DCs expressing fully active IDO enzyme.

In spite of this controversy, there are strong evidences that some populations of DCs express IDO conferring them immune suppressive capacity [33, 34, 40, 107], which can suppress T-cell proliferation in an allogeneic mixed lymphocyte reaction [100] and induce regulatory CD4+ cells [114]. These IDO expressing DCs behave in a dominant fashion, that is, they can suppress T-cell response to antigens presented by neighboring IDO negative cells. This effect has been demonstrated *in vitro* using simultaneously IDO negative and positive DCs in a mixed lymphocyte reaction [115]. This dominant suppressive effect can be crucial in cancer hosts, where the presence of IDO positive DCs in lymph draining nodes could induce suppression in the surrounding media thus facilitating the tumoral metastasis [115, 116].

Other enzymes of the kynurenine pathway are also induced during maturation of DCs. Studies with microarrays have shown that maturation of human DCs with TNF-α and polyI:C induces coordinately in less than 24 hours a cluster of genes involved in Trp metabolism: IDO, kynureninase, kynurenine monooxygenase, and tryptophanyl tRNA synthetase [31]. The final Trp degrading enzyme 3-hydroxyanthranilic acid oxidase that produces quinolinic acid is downregulated in these conditions. Recently, Belladonna *et al.* have shown that murine splenic DCs express all the enzymes necessary to synthesize quinolinic acid, a reaction that is enhanced by the addition of IFN-γ [117]. Murine CD8-DCs subsets do not have IDO activity, but when these cells are stimulated with IFN-γ in presence of kynurenine, these cells acquire suppressive properties. This implies that, independently of Trp

concentrations, DCs can be induced to create tolerance if there is enough supply of kynurenines.

The possibility of generating DCs expressing active IDO or even other enzymes of the kynurenine pathway is an important point as DCs have been used in cancer immunotherapy trials, and generating tolerogenic DCs could lead to opposite results than the expected ones. In our experience, clinical grade DCs generated with standard protocols with GM-CSF+ IL-4 and matured with TNF-α and poly I:C do not express active IDO (unpublished observations). Nevertheless, when using DCs in clinical trials it would be wise to check IDO expression by measuring Trp consumption and the kynurenine concentration in the culture supernatants.

9.3. Mechanism of Suppression

Since the discovery of the role of IDO as an immunosuppressive molecule [2], several hypotheses have arisen to explain its mechanism of action. The initial theory proposes that Trp depletion in the T-cell surrounding microenvironment causes functional suppression of lymphocytes [32]. This hypothesis has been recently critically discussed [113, 114], because there are some caveats to explain immunosuppression, particularly *in vivo*. Other hypotheses suggest that Trp metabolites, mainly kynurenine, induce suppression [104]. Finally, the third hypothesis is that Trp metabolism modifies APCs properties [32, 118]. Probably this third mechanism is indirect and mediated by the first two. Independently of the mechanism involved, an important fact is that it is initiated by an antigen-specific response, so it is not a general immunosuppression. Moreover, most likely, these mechanisms are not mutually exclusive and can be synergistic, and in some situations suppression may be mainly the result of elevated kynurenine metabolites rather than the depletion of Trp, and vice versa. However further research is needed to clarify these possibilities, particularly to define precisely the concentration of Trp in the microenvironment and the distribution of the Trp metabolites that can have different functions.

9.3.1. *The Trp Depletion Theory*

IDO induction in allogeneic mixed lymphocyte reaction provoked Trp depletion, driving to T-cell arrest. Supplementation with high Trp concentration to IDO+ macrophages prevents suppression [101]. As a possible explanation of the mechanism involved, Munn *et al.* [119] proposed the conserved amino acid sensitive GCN2 kinase pathway as the molecular target of IDO suppression in T cells. Trp depletion causes a rise in uncharged tRNA in T cells, and GCN2 contains a regulatory domain that binds the uncharged forms of tRNA. As a result, there is an activation of GCN2 kinase in T cells that initiates a highly conserved downstream signaling pathway,

termed integrated stress response. This pathway leads to T-cell anergy. However, Fallarino *et al.* [114] showed that it is not Trp depletion alone, but the combined action of Trp depletion and kynurenines that is necessary to downregulate T-cell CD3ζ expression through a GCN2 kinase-dependent mechanism. Furthermore, some authors have found that T cells can proliferate in Trp-deprived medium [103].

To achieve an efficient T-cell suppression, the concentration of Trp in the medium should be below 1 μM [101]. IDO is an intracellular enzyme with cytoplasmic location [120], and macrophages need a very efficient transport system to import Trp from the surrounding medium (Fig. 4) [121]. There is a widely distributed amino acid transporter system for Trp and other neutral amino acids, named L-system. This L-system has a K_m for Trp of 23.6 μM, which is too high to decrease the concentration of this amino acid to the extent needed to suppress T cells [28]. However, during monocytes

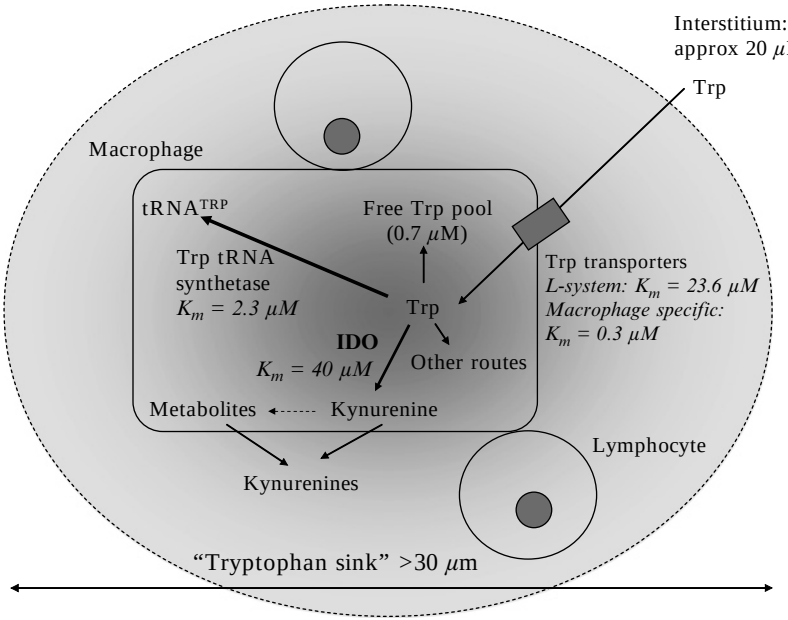

FIG. 4. Generation of an immunosuppressive environment by antigen-presenting cells (APCs). Trp is internalized from the medium by two types of transporters: A L-system, and another more specific of macrophages and with a higher affinity for Trp. Inside the cell, Trp is derived for the synthesis of proteins and transferred to a tRNA by a Trp tRNA synthetase, or directed to the kynurenine pathway by indoleamine 2,3-dioxygenase (IDO), which is the first enzyme of this route, to be further metabolized to different kynurenines. Both the kynurenines and Trp depletion can provoke arrest of activated T cells.

differentiation to macrophages, another transporter with high affinity for Trp is highly induced [122], which is not expressed in T cells. This transporter expressed in macrophages has a K_m for Trp of 0.2–0.3 μM, so it has the capacity to efficiently import Trp in the range of amino acid concentrations necessary to produce T-lymphocyte arrest. Considering that the K_m of the IDO enzyme is 40 μM for l-Trp [21], this enzyme works at suboptimal substrate concentration. This implies that the velocity of Trp degradation decreases as the concentration of Trp diminishes.

Neither the amino acid transporter nor the IDO are polarized during the synaptic contact between T lymphocytes and IDO expressing cells. For this reason, the proposed "Trp sink" is not limited to the boundary between these cells, but must measure at least double of the average T-lymphocyte size (Fig. 4). This point is challenged by the observation made by Terness *et al.* [113] that a local Trp decrease could be quickly compensated by gradient diffusion from the surrounding tissue and plasma, a flow that it is particularly intense in human placenta [123, 124]. Also, positron emission tomography shows that amino acids diffuse in few minutes from the vascular compartment to irrigated tumors, but not to poorly irrigated or necrotic tumors.

9.3.2. *Immune Modulatory Function of Kynurenine Pathway Metabolites*

Trp metabolism produces different kynurenines, some of them with important tolerogenic properties; the provoked suppression can be specific for certain T-cell subpopulations: 3-hydroxyanthranilic acid and quinolinic acid induce apoptosis in murine thymocytes and TH1 cells, but not in TH2 cells [125]. The apoptosis is mediated by caspase-8 and the mitochondrial release of cytochrome C. This suppression is also observed in human T cells, showing that kynurenines suppress irreversibly allogeneic T-cell proliferation *in vitro* [104]. l-Kynurenine inhibition of proliferation occurs only at the early stages of cell activation [103], which is in accordance with previous data showing that macrophage-dependent arrest of T cells specifically affects the initial transition from quiescence to proliferation [102]. These Trp catabolic products exert a cytotoxic action on CD3+ cells, mainly when activated. B and NK cells are also susceptible to these compounds [104].

As we have mentioned before that Trp addition can reverse IDO-mediated macrophage suppression [101], which apparently rules out significant contributions from kynurenines since more Trp yields more metabolites, yet it restores T-cell proliferation [126]. Enzymes of the Trp metabolism induced during DCs maturation [31] lead to an accumulation of kynurenine, 3-hydroxykynurenine, and 3-hydroxy-anthranilate. The concentration of individual kynurenines needed to achieve suppression is higher that those that can be found *in vivo*, but when there are several kynurenines together in

the medium the suppression is achieved at more physiological concentrations [104]. DCs are more resistant to the toxic actions of kynurenines than lymphocytes and NK cells [104, 125]. Undoubtedly, the increase of Trp metabolites is accompanied by a decrease in the concentration of this amino acid. Apart from their cytotoxic effect, kynurenines cooperate with Trp depletion in developing a tolerogenic microenvironment affecting T-cell response capacity. The suppressive effect of l-kynurenine and picolinic acid on T- and NK-cell proliferation is potentiated by Trp depletion [103]. IDO expressing DCs mediate activation of GCN2 kinase in peripheral CD4+ CD25− cells, which participate in acquiring a regulatory phenotype Foxp3 +. This effect can be mimicked by stimulating CD4+ lymphocytes in a conditioned medium low in Trp and with kynurenines. This suggests a relevant role of toxic Trp metabolites in DCs-induced suppression.

10. Protective Role of IDO in Rejection of the Fetus During Pregnancy

Fetus can be considered from an immunological point of view as a semi-allogeneic transplant; having half of the genes from the father, fetus should be rejected by the maternal immunocompetent system. Nevertheless, evolution has found the mechanism to avoid this attack by maintaining the general immune competence. This is one of the most intriguing questions in biology. In 1998, Munn *et al.* showed the implication of IDO in protecting fetus from the maternal attack in a mouse model [2]. The administration of the IDO inhibitor, 1-methyl-Trp, to pregnant mice during allogeneic gestation increased the rate of abortion of allogeneic fetuses a few days after implantation. The blockade of IDO permits the recognition of fetal antigens by T cells, provoking an intense inflammatory response with antigen-independent complement deposition and hemorrhage [127]. In syngeneic pregnancy, where the fetuses have the same antigens as the mother, this IDO inhibitor did not have any effect and the fetuses developed normally. The immunology of placentation [128] and the development of pregnancy is different between human and mouse, and we have to be cautious when extrapolating result from mice experiments.

In humans, IDO is produced as early as day 6 by blastocysts, and thereafter throughout pregnancy by syncytiotrophoblasts, extravillous cytotrophoblasts, and macrophages in the villous stroma and in the fetal membranes [129]. This enzyme is induced by the hormones that are highly produced during pregnancy, such as chorionic gonadotrophin [130]. IDO enzyme is fully active as demonstrated in the cultures of placenta explants [129], producing local Trp depletion and kynurenines production. Furthermore, some

authors have found decreased serum Trp concentration during pregnancy accompanied by an increase in the kynurenine/Trp ratio, which suggests an increased IDO-dependent Trp degradation. However, the degree of change of these compounds is not very high and there is a great overlap between different situations. At the end of pregnancy, there is a lower serum kynurenine concentration due to a lower Trp concentration.

The activation of the kynurenine pathway can affect the mood of the mother after delivery. Trp is the precursor of the neurotransmitter serotonin, whose synthesis could be compromised by Trp availability during pregnancy. As a result of the activation of the kynurenine pathway, there is a decrease of Trp, particularly at the end of term and in the early puerperium. Several studies have demonstrated that depression and anxiety symptoms in the early puerperium are associated with an increased catabolism of Trp [132].

As the kynurenine pathway seems to play an important role in pregnancy as an immunosuppressive mechanism, a defect in the activation could lead to complications and even abortion. As it has been mentioned before, ligation of CTLA-4 expressed by T-regulatory cells to the B7 molecules on DCs from normal pregnant women induces IFN-γ-dependent IDO expression, but is decreased in DCs from women suffering spontaneous abortion [133]. Preeclampsia is an important complication that occurs during the second half of pregnancy, a condition characterized by a relative failure of the uteroplacental circulation. This occurs as a result of an exacerbated inflammatory activity, that also happens, but with much lower intensity, in normal pregnancy. There are evidences that IDO may impair the normal maternal systemic inflammatory response during normal pregnancy, and this inhibition becomes disrupted in preeclampsia [131]. The activity and mRNA expression of IDO in term placentas is lower in preeclampsia compared to normal pregnancy, which is reflected by lower plasma kynurenine/Trp ratios [131].

However, Trp degradation pathway is not the only mechanism that protects the fetus from immune maternal attack. Mice lacking Indo gene can deliver allogeneic and syngeneic puppies with similar success rates, and 1-methyl-Trp does not affect pregnancy outcome in these knockout mice [134]. These data call for the existence of other controls of both innate and adaptive immunity during pregnancy. There are other suppressive molecules expressed in placenta, such as the nonclassical MHC class I antigen HLA-G [135], probably providing redundant mechanisms to create a protective microenvironment. Both HLA-G and IDO suppress T-cell response by independent and complementary mechanisms [136]. In fact, there is a relationship between IDO and HLA-G; we have shown that 1-methyl-Trp increases the expression of HLA-G in APCs [137], leading to decreased NK cytotoxicity and T-cell proliferation [138].

11. Clinical Implications of the Immunosuppression Provoked by the Kynurenine Pathway

11.1. Cancer Immune Evasion by IDO Expression

Tumor cells have great plasticity and employ diverse immunosuppressive tools to escape from the host attack, which are common to different cancer cell types independently of the acquired oncogenic mutations [139]. Furthermore, very likely, tumors employ the machinery used by the fetus to avoid maternal rejection. One of these tolerogenic mechanisms is the expression of IDO and production of kynurenines. Certainly, as early as in 1956, Boyland and Williams [140] observed increased Trp metabolism with kynurenine excretion in patients with cancer. In addition, the expression of IDO and kynurenine production has been shown in many different human tumor cell lines exposed to IFN-γ [3, 141]. Positron emission tomography has demonstrated increased Trp captation by brain tumors, which metabolized this amino acid to kynurenine [142].

After the discovery of the immunosuppressive role of IDO in pregnancy, Mellor and Munn soon postulated that tumor cells could employ this enzyme to evade the immune system attack [23]. Evidence of this new hypothesis came initially from the use of the IDO inhibitor 1-methyl-Trp, which increases T-cell attack against tumors. This compound can increase the allogeneic T-cell response to Lewis lung carcinoma cells when present in the medium; and *in vivo* administration caused growth delay in syngeneic mice [143]. An obvious question is: can depletion of a nutrient be beneficious for tumoral growth? The answer comes from the hypothesis of cancer immune editing [144]. Trp depletion can negatively affect tumor growth [3], but if tumor cells were less sensitive to Trp deprivation than T lymphocytes, this nutrient shortage could give a notorious advantage to their survival. Cancerous cell elimination by immune cells could make a selective pressure where some tumor cells express IDO enzyme helping to evade tumor surveillance [145]. As a result, IDO expression can be beneficial for the tumor even if it was initially noxious, due to its role as an immunosuppressive molecule [146]. The expression of IDO in tumor cells has been shown to be an independent prognostic factor of impaired survival in patients with serous advanced ovarian cancer [147] or colorectal cancer [148]. In endometrial cancer, IDO expression positively correlates with surgical stage, myometrial invasion, lymph-vascular space involvement, and lymph node metastasis, but not with the histological grade. Patients with high IDO expression have significantly impaired overall survival and progression-free survival [149]. If the induction of IDO in tumor cells is also kept under the control of IFN-γ, then it can be turned on precisely when activated lymphocytes are approaching.

Uyttenhove *et al.* [139] have demonstrated variable IDO expression in primary human tumors: while being high in prostate, colorectal, pancreatic, and cervical tumors, it is almost absent in choriocarcinomas and testicular seminomas. By elegant experiments, this group demonstrated the role of IDO in tumor evasion: transfection of the immunogenic mouse tumor cell line P815 with IDO made it resistant to rejection even in preimmunized mice, and systemic treatment of mice with 1-methyl-Trp delayed tumor outgrowth. Interestingly, the effects observed with this IDO inhibitor were dependent on the presence of functional T cells.

Bin1-Amphiphysin2 is a cancer suppressor gene that participates in the traffic control of vesicles and in signal transduction [150, 151], and the lack of Bin1 expression in some cancers is related to the expression of Indo gene [150]. When Bin1 is repressed, IFN-γ increases STAT1 and NF-κB-dependent Indo transcription provoking an overproduction of IDO protein, which favors the immune escape of the tumor cells [150]. The enzyme cyclooxygenase-2, highly expressed in many tumors [152], is another possible inductor of IDO [153], as cyclooxygenase-2 inhibitors suppress IFN-γ-mediated IDO expression [154]. It was demonstrated in a mouse model of breast cancer that administration of the cyclooxygenase-2 inhibitor celecoxib decreased IDO expression and improved the response to the tumor. Furthermore, there was a direct correlation between cyclooxygenase-2 and IDO expression in primary human breast cancer specimens, although the clinical significance has not been established yet [153].

11.1.1. *APCs in Tumor Draining Lymph Nodes Can be Induced to Express IDO*

Even though some tumor cells can express IDO suppressing activation of T cells in the tumoral microenvironment, there is still capacity to develop an immune response outside the tumor itself [155]. However, the tumoral microenvironment can induce the expression of IDO in infiltrating APCs, mainly DCs, which could be an efficient mechanism to overcome the adaptive immunity allowing tumor survival [126]. The presence of IDO positive infiltrating cells has been demonstrated immunohistochemically in some tumors such as hepatocarcinoma [156], breast, colon [40], and nonsmall cell lung carcinoma [157]. In the latter case, the infiltrating cells expressing IDO were eosinophil granulocytes.

It is well known that the analysis of the tumoral cells in the sentinel lymph node in patients with cancer is an important aid in the prognosis and therapeutic approach [158]. Additionally, the presence of plasmacytoid DCs expressing IDO in tumor draining lymph nodes has been reported in patients with melanoma [116], even in absence of tumoral cells. The immunohistochemical detection of IDO expressing DCs in the sentinel node at the

moment of diagnosis indicates adverse prognosis in this disease [115]. These mononuclear cells expressing IDO present in tumor draining lymph nodes may confer an advantage to the tumor to escape the immune response.

11.1.2. *Measurement of the Kynurenine Pathway in Patients with Cancer*

An increased Trp catabolism in both solid and hematological cancers, reflected by decreased Trp concentration or, less frequently, by increased kynurenine levels has been documented. Probably the increased kynurenine formation is associated with an ineffective activation of the immune system as it is usually associated with increased neopterine levels and bad prognosis [159, 160]. Indeed, lower Trp/kynurenine ratio correlates in some studies with the deterioration in quality of life [159, 161, 162]. Examples of tumors where the kynurenine pathway is frequently activated are carcinomas of bladder [163], colon, and breast [164] as well as melanoma [160] and lymphomas [164]. In a study of patients with gynecological cancer, it was shown that there was a decrease of circulating Trp but not kynurenine, compared to healthy controls [165]. Patients with disseminated melanoma have lower serum Trp concentration than the cancer-free population [160].

Treatment of cancer with biological modifiers can induce this immunosuppressive pathway, potentially with negative effects in the clinical outcome. For example, patients undergoing a phase I toxicity trial of recombinant IL-2 had decreased plasma Trp levels and corresponding increased urinary kynurenine and neopterin, indicating activation of the immune system and, particularly the kynurenine pathway [166]. The treatment of patients with advanced malignancies in a phase I trial with TNF and IFN-γ therapy provoked a clear increase of IDO in monocytes, accompanied with kynurenine and neopterin excretion in urine [167].

11.1.3. *Effect of the Combination of IDO Inhibitors with Chemotherapeutic Drugs*

As it was indicated before, both tumors and APCs, either spontaneously or after cytokine stimulation, can express IDO, which can drive to immune suppression. For this reason, therapeutic approaches to treat cancer patients should overcome the tolerance molecular tools that tumor cells employ.

IDO inhibitors slow down tumor growth [139, 143, 150], but a complete remission was achieved with a combination of an IDO inhibitor and classical chemotherapeutic drugs, as demonstrated by Muller *et al.* in a mouse model [150]. The benefit of this therapeutical approach seems to be based on the activation of the immune system and on the toxicity of the drug. The effectiveness of this therapeutic approach requires an intact T-cell response, as CD4+ depletion impairs the effectiveness of the treatment.

As the aim of immunotherapy is to use the adaptive immune system to provoke an effective response to tumor cells, the IDO enzyme can be a therapeutic target to potentiate T-cell response in cancer immunotherapy [32]. An immune therapeutic approach to treat tumors is the use of DC-based cancer vaccines. DCs used for therapeutic vaccination in melanoma patients express IDO, and can attract or induce FoxP3+ regulatory T cells at the site of application [168]. The combination of this treatment with drugs that inhibit IDO has provided encouraging results reducing primary tumor burden, preventing metastasis, and increasing survival in a mouse model of breast cancer [153]. Different molecules are being tested as inhibitors of IDO looking for a favorable pharmacokinetics and efficiencies [150, 169].

As we have mentioned before in the case of pregnancy, the kynurenine pathway probably is not the only mechanism that tumors use to evade the immune system. The knowledge of how tumor cells actively and passively evade the immune system will provide in the next future new, and probably safer, therapeutic approaches to treat patients with advanced cancer [150].

11.2. Autoimmune Diseases

A clear failure of the mechanisms of self-tolerance occurs in autoimmune diseases, where T and/or B lymphocytes react against their own organism causing tissue damage. This is provoked by alterations in the equilibrium between immune activation and suppression. We will show some examples where the kynurenine pathway seems to play a role, mainly in the treatment of these diseases:

1. Multiple sclerosis is a disease characterized by relapsing and remitting T-cell-mediated autoimmune inflammation of the central nervous system (CNS). The activation of the kynurenine pathway seems to play a role in the remission of this disease, thus controlling the clinical course of the disease. IFN-β treatment is usually employed in relapsing-remitting multiple sclerosis patients and provokes an increase in the kynurenine/Trp ratio [170]. Experimental autoimmune encephalomyelitis is a murine model of the disease, where it has been observed that IDO expression by macrophages/activated microglia contributes to remission [171, 172], but mice lacking the IFN-γ receptor did not recover from the disease [173]. During the recovery phase, the higher IDO expression is reflected by an increased serum kynurenine/Trp ratio [171]. Also, 1-methyl-Trp exacerbated symptoms of the disease in this animal model [171, 172]. Myelin-specific T cells are suspected to cause multiple sclerosis, and when stimulated with tolerogenic altered self-peptides express IDO, which can have suppressive properties [174]. Certainly, several kynurenines suppressed proliferation of myelin-specific T cells and inhibited production of proinflammatory TH-1 cytokines [174]. The

synthetic analog of kynurenines, *N*-(3,4,-dimethoxycinnamoyl)anthranilic acid, not only has these actions *in vitro* but also, more importantly, oral treatment greatly ameliorated experimental autoimmune encephalomyelitis in mice.

2. Rheumatoid arthritis is a destructive and painful autoimmune disease that attacks the joints. In rheumatoid arthritis, there is an activation of the kynurenine pathway [175], whose importance has been shown by the demonstration that 4–1BB antibodies amelioration of rheumatoid arthritis in a mouse model is through the IFN-γ-dependent IDO induction [58]. The recombinant CTLA-4-Ig fusion protein (abatacept) has been approved to treat autoimmune rheumatoid arthritis, and the clinical benefits can be related to the capacity of this molecule to induce IDO-mediated immunosuppression, as we have described in previous paragraphs.

3. Type 1 diabetes mellitus is a T-cell-mediated autoimmune disease that results in the destruction of the insulin-producing β cells of the pancreatic islets of Langerhans. Transplantation of isolated human islets is a possible treatment for human diabetic subjects, although it is necessary immunosuppressive therapy to avoid both recurrence of autoimmunity and allorejection [176]. NOD mouse is a model of autoimmune disease where there is a predisposition to develop type 1 diabetes as a result of a deficiency of both peripheral and central tolerance. Adenoviral gene transfer of IDO to prediabetic NOD mouse pancreatic islets allows the prolongation of islet graft survival into NODscid recipient mice [177]. However, this treatment does not impair the onset of the disease. Apart of the benefits of IDO induction in ameliorating the disease, NO-mediated disruption of the IDO-inducing pathway can provoke type 1 diabetes mellitus in NOD mice [88].

11.3. Transplantation

As it has been described before, the IDO enzyme, via Trp depletion, suppresses adaptive T-cell responses in inflammation, host immune defense, and maternal tolerance. However, its role in solid organ transplantation remains unclear. Several groups have investigated the role of IDO expression in different models of transplantation. A possible role for IDO in modulating the response to a graft was first suggested by the finding that overexpression of IDO in donor pancreatic islets prior to transplantation extended survival in an animal model [177]. In rats, it has been described that IDO-induced Trp metabolites (kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid) suppress the T-cell response and prolong skin allograft survival [178].

Also, data obtained from studies of endothelial cells in graft rejectionsuggest that the variable expression of IDO in different endothelial cells is

important not only in the regulation of graft rejection but also could be a potential therapeutic strategy [179, 180]. In addition, available experimental data indicate that IDO may participate in the mechanism of spontaneous donor-specific tolerance of liver grafts [181]. Finally, a recent report found upregulation of IDO in renal biopsies from rejection episodes, a finding absent in nonrejected grafts [182], probably associated with immune activation. Moreover, acute rejection in patients after kidney transplantation is associated with increased serum and urinary kynurenine/Trp ratio.

In summary, based on these findings it appears that cells expressing IDO can inhibit T-cell responses and hence induce tolerance in some models of transplantation. Further studies will be needed to evaluate whether the kynurenine/Trp could be useful in monitoring allograft rejection.

11.4. HIV Immunoescape by Inducing Kynurenine Pathway

Usually after a viral infection, most viruses are either eliminated or suppressed, persisting as a low-damaging latent infection. After infection with HIV, there is a cytotoxic T-lymphocyte and antibody response, but the virus is not definitively eliminated [183]. HIV-1 infection can persist without clinical manifestations for years, despite an apparently functioning host immune system. The virus can induce some tolerogenic mechanisms that may protect itself from immune attacks, such as FAS-ligand expression or downregulation of MHC. Regulatory T cells expressing high levels of CTLA-4 and with suppressive activity are increased in tissues from patients suffering HIV infection and SIV-infected macaques. The administration of antibody that blocks CTLA-4 in SIV-infected macaques treated with antiretroviral therapy decreased IDO expression and the level of the suppressive cytokine TGF-β in tissues [184]. An important effect is that CTLA-4 blockade was associated with decreased viral RNA levels in lymph nodes and increased effector function of both SIV-specific CD4+ and CD8+ T cells. Also, some HIV strains can induce IDO in macrophages [185] and microglia [186], and the immunosuppressive properties of IDO could help virus to avoid cellular immunity. As IDO-expressing infected macrophages are protected from the immune system, this could also serve as a stable viral reservoir. For this reason, pharmacological modulation of this enzyme could help in therapy, as in a murine model, IDO inhibition with 1-methyl-Trp facilitates cytotoxic lymphocyte response against virus-infected macrophages [187].

The kynurenine pathway can also influence the low hemoglobin levels found in HIV-1-positive patients [188]. It is rather speculative, but probably, that Trp degradation arrests proliferation of not only T cells but also other

cells such as erythroid progenitor cells [188]. In addition to participating in the immune evasion of the virus, the kynurenine metabolite quinolinic acid can mediate in the neurological pathogenesis of the disease. Quinolinic acid is an endogenous agonist of the *N*-methyl-D-aspartate receptor, a subtype of the glutamate receptor in brain. Macrophages infected with HIV-1 produce quinolinic acid, and therefore provoke neuronal dysfunction in these patients. Elevated levels of quinolinic acid associated with the severity of neurological symptoms have been found in the CSF of patients with AIDS-related dementia complex [189].

Determination of Trp and kynurenine in serum reflects the activation of the kynurenine degrading pathway in this disease [190–192], and the ratio correlates with the serum concentration of IFN-γ [190]. Patients infected with HIV show increased kynurenine/Trp ratio, which is more noticeable in symptomatic patients [190]. This ratio reflects the stage of the disease and correlates with CD4+ count, the classical parameter of evaluation AIDS patients [191]. Treatment with antiretroviral therapy increases Trp and decreases kynurenine concentrations [192]. It remains to be explored whether treatment with antiretroviral drugs inhibits IDO and other enzymes of the kynurenine pathway.

11.5. Cardiovascular Diseases

Inflammation and immune activation play a role at all stages of the pathogenesis of atherosclerosis and cardiovascular disease. Accordingly, inflammation markers are elevated in patients with clinical and subclinical atherosclerosis and predict cardiovascular events. Interestingly, enhanced Trp degradation and increased serum kynurenine/Trp ratio has been reported in patients with coronary heart disease compared with healthy controls, indicating an activated cellular immune response. The enhanced Trp degradation in these patients has been correlated with enhanced neopterin formation [193].

Macrophages are critically involved in plaque formation and in this cell type IFN-γ induces IDO activity, which degrades Trp via the kynurenine pathway. IFN-γ is a central player in atherogenesis and in the development and progression of cardiovascular disease. It appears that in cardiovascular disease, biochemical reactions induced by IFN-γ may have detrimental consequences for host cells [194]. Finally, in a porcine model of cardiac arrest, the potential use of the Trp degradation rate as a means of estimating the extent of immune activation was evaluated. Results suggested that the kynurenine/Trp ratio could serve as a marker of immune activation [195].

11.6. Neurological and Psychiatric Diseases

Several lines of evidence suggest a link between the immunological network and neuroendocrine functions with consequences in the psychological status of patients. In fact, in chronic inflammatory diseases, mood disorders and related symptoms are observed. These are also observed as adverse effects of the administration of cytokines such as IFN-α and IL-2 that are employed to treat pathologies, such as hepatitis C and cancer. Intriguingly, the most serious side effects are symptoms associated with depression: fatigue, drowsiness, irritability, loss of appetite, and cognitive deficits. Finally, in animals, the administration of proinflammatory cytokines induces changes in the behavior that resemble the vegetative symptoms of depression in humans.

The neurotransmitter serotonin is synthetized by the tetrahydrobiopterin-dependent Trp 5-hydroxylase and appears to play a role in depression symptomatology. In depressed people, changes in serotonin and its receptors have been described. In addition, evidence exists for a dysregulation of the noradrenergic system and a hyperactive hypothalamic–pituitary–adrenal axis in depression:

1. Trp is a source of serotonin generation. Consequently, in states of persistent immune activation, availability of Trp is decreased due to the IDO activity and leads to reduced serotonin production and serotonergic dysfunction [196, 197]. Indeed, increased degradation of Trp and low serum levels of Trp correlate with neuropsychiatric abnormalities such as cognitive decline and depressive symptoms especially in long-lasting chronic diseases [199]. But also, increased degradation of Trp has been demonstrated in patients with depression [196]. This same mechanism is responsible for the depressive states associated with cytokine therapy and those patients developing more pronounced depressive symptoms show a more marked increase in Trp metabolism [200].

2. Another mechanism that may contribute to the development of neurologic/psychiatric disorders is the accumulation of neuroactive kynurenine metabolites such as quinolinic acid, which is an *N*-methyl-D-aspartate (NMDA) receptor agonist in situations of Trp depletion [201]. An increase in quinolinic acid is associated with features of depression [189].

3. Proinflammatory cytokines by stimulating IDO lead to Trp depletion, and thus affect serotonin metabolism. Also, IL-1, IL-2, and TNF-α influence noradrenergic activity and IL-1, IL-6, and TNF-α are stimulators of the hypothalamic–pituitary–adrenal axis. Altogether, administration of cytokines may induce alterations in the brain resembling those found in depressed patients, which leads to the hypothesis that cytokines induce depression by their influence on the serotonin, noradrenergic, and hypothalamic–pituitary–adrenal system [202, 203]. In summary, activation of IDO appears to be an

important link between the immune system and the pathogenesis of depression.

Impairment in dopaminergic neurotransmission is crucial in the pathogenesis of schizophrenia, suggesting a disequilibrium between dopaminergic and glutamatergic neurotransmission. The immune system may have effects on glutamatergic neurotransmission mediated by the kynurenine pathway. Glutamatergic hypofunction is mediated by the NMDA-receptor antagonism. The only endogenous NMDA-receptor antagonist identified is kynurenic acid. This metabolite also blocks the nicotinergic acetylcholine receptor and increased kynurenic acid levels can explain psychotic symptoms and cognitive deterioration [204].

In schizophrenia, there is a shift to increased type 2 immune response with two functional consequences: downregulation of the expression of IDO (located in astrocytes and microglial cells) while TDO is upregulated. Also, the TH1/TH2 imbalance is associated with a high activation of astrocytes and imbalance in the activation of astrocytes/microglial cells, which leads to accumulation of kynurenic acid. This happens because astrocytes lack the enzymatic machinery for the normal metabolism of Trp and further production of quinolinic acid. Thus, kynurenic acid accumulates in the CNS, while the metabolic pathway in microglial cells is blocked. Accordingly, an increase in TDO activity has been observed in critical CNS regions of schizophrenics. These mechanisms result in an accumulation of kynurenic acid in critical CNS regions of schizophrenics compared to controls [205]. Thus, the immune-mediated glutamatergic–dopaminergic dysregulation may lead to the clinical symptoms of schizophrenia.

12. Conclusions

Since the discovery of IDO in maintaining pregnancy in mice [2], considerable amount of evidence has shown that the kynurenine pathway induces an active tolerogenic state, helping in the regulation of the quality and intensity of the specific immune response [32]. There is a tight regulation of IDO expression that depends on the multiple incoming signals and also on the capacity of a functional immune system to decide whether this enzyme is active or not. The effect is not restricted to a localized microenvironment but can act at distance and maintaining the specificity by means of regulatory T cells and tolerogenic DCs [32]. However, the mechanism of immunosuppression and the type of DCs that can express IDO should be further clarified [112, 113]. Alterations of this pathway have been implicated in some diseases such as autoimmune diseases. The discovery that tumor cells can use this

metabolic route as a mechanism to evade the immune attack [206] opens a new perspective in the treatment of this disease. For example, drugs that inhibit this pathway emerge as a type of immunomodulator that would help conventional drugs to treat tumors. Very promising are the results obtained

TABLE 1
CLINICAL IMPLICATIONS OF THE KYNURENINE PATHWAY

Disease		Evidence in humans	Mediator	References
Cancer		Tumor cell lines	↑IDO, ↑Kyn	[3, 140]
	Brain	Yes	↑Trp captation	[141]
	Lewis lung carcinoma	Yes	1MTrp increases T cell response	[143]
	Ovary	Yes	↑IDO	[147]
	Colorectum	Yes	↑IDO	[148]
	Melanoma	Yes	↑Kyn	[160]
	Bladder	Yes	↑Kyn	[163]
	Breast, lymphoma	Yes	↑Kyn	[163, 164]
Autoimmune diseases	Multiple sclerosis	Yes	↑IDO, ↑Kyn/trp	[170–172, 174]
	Rheumatoid arthritis	Yes	↑Kyn/trp	[175]
	Type 1 diabetes	No		[88, 176, 177]
Transplantation	Pancreatic islets	No	Overexpression of IDO	[177]
	Rat skin allografts	No	↑Kyn	[178]
	Graft rejection	Yes	IDO	[179, 180]
	Liver graft	Yes	IDO	[181]
	Kidney transplantation	Yes	↑IDOkyn/trp relates to rejection	[182]
AIDS		Yes	↑IDO leads to virus immune escape ↑Kyn/trp	[186, 188–192]
Cardiovascular diseases	Coronary heart disease	Yes	↑Kyn/trp	[193]
	Cardiac arrest	No	↑Kyn/trp	[195]
Neurological and psychiatric diseases	Depression	Yes	↓Trp ↑Quinolinic acid ↑Proinflammatory y cytokines	[196, 198, 202, 203]
	Schizophrenia	Yes	↑Kynurenic acid	[204, 205]

by Muller *et al.* [150] combining IDO inhibitors and conventional cytotoxic drugs. The results obtained by cancer vaccines, that are nowadays rather limited, would also be improved by the use of IDO inhibitors. Considering how important this pathway is in the diseases, some of them mentioned in this chapter (summarized in Table 1), it is very likely that the measurement of the degree of activation of this pathway will be introduced in the routine in clinical laboratory in the near future [207].

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PATHOPHYSIOLOGY OF TUMOR-ASSOCIATED MACROPHAGES

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1. Abstract

The macrophage is an important component of the human immune defense mechanism. Cancer cells secrete a variety of chemoattractants that attract macrophages and cause them to accumulate in the tumor tissue, wherein the

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macrophage becomes a tumor-associated macrophage (TAM). Recent evidence has shown that the function of tumor stromal TAMs can be modified by cancer cells and the factors they secrete. TAMs are directed toward stimulating tumor growth and progression and thus have protumorigenesis activity. However, there is also limited evidence that TAMs still play an important role in the killing and destruction of cancer cells, inhibit cancer metastasis, and have antitumor activity. Whether TAMs show protumorigenesis or antitumor activity depends on interaction with cancer cells, other stromal cells, and the tumor microenvironment. Gene expression profiles of TAMs, cancer cells, and other stromal cells are altered by cell–cell interactions. This phenomenon results in positive or negative regulation of angiogenesis, tumor cell proliferation, apoptosis, cancer cell migration and invasion, and the secretion of a variety of cytokines or factors. Whether TAMs have a positive or negative effect also depends on the macrophage activation state, the status of tumor development, and the anatomic locus of macrophage infiltration. Understanding of the mechanisms that regulate TAM function is essential in designing therapies to promote antitumor activity in humans. Although limited evidence from both animal and human studies indicates a potential role for TAMs in cancer treatment, the clinical usefulness of these therapies require further studies.

2. Introduction

Cancer progression is a complex multistep process that consists of transformation, tumor growth, invasion, and metastasis. Recent evidence shows that the stromal extracellular matrix and stromal cells (including fibroblasts, inflammatory cells, and endothelial cells) play an important role in promoting tumor progression [1]. Cancer progression is therefore not exclusively regulated by the disruption (overexpression or underexpression) of oncogenes and tumor suppression genes in cancer cells, but also depends on the stromal compartment to create a more tumor-promoting microenvironment. The interaction between cancer cells and stromal cells has been recently shown to promote tumorigenesis [1, 2].

It is now becoming clear that the inflammatory cells present in the tumor microenvironment play an indispensable role in the cancer progression. Substantial evidence suggests that stromal cells adjacent to cancer cells, including fibroblasts and inflammatory cells, such as macrophages, neutrophils, and lymphocytes, can interact with the cancer cells and express angiogenic factors [3–5].

The macrophage is a major component of the stromal cells around tumors. Initially, these macrophages were considered to have a cytotoxic effect after

activation by a variety of stimulators and to play an important role in immune defense against microbiologic pathogens or cancer cells. However, recently, tumor-associated macrophage (TAM) function was shown to be modified by cancer cells and to be switched to facilitate tumor growth and metastasis. Furthermore, recent studies have also demonstrated that interactions occur between TAMs and cancer cells and between TAMs and other stromal cells, and that these interactions change the gene expression profile of TAMs, cancer cells, and other stromal cells. In this chapter, we will focus on the protumorigenesis (stimulating tumor progression) and antitumor effects of TAM and the effects of the interactions between TAMs and cancer cells or other stromal cells on the regulation of the expression of genes that are responsible for the tumor progression or tumor inhibition. Finally, we will discuss the clinical implications of using activated macrophages or gene therapy to activate TAMs in the treatment of human cancers.

3. Association Between TAM Density and Patient Prognosis

The macrophage is an important component of the inflammatory cells within the tumor stroma. Evidence suggests that macrophages may account for the major part of the host leukocyte infiltrate in the majority of human malignant tumors [3]. Several investigators had counted the macrophage number in cancer surgical specimen under microscopy by immunohistochemical staining, and they reported that the percentage of macrophages in human malignancies is between 10% and 65%. These macrophages are referred to as TAMs. TAMs are mainly derived from peripheral blood monocytes, which are recruited into the tumor mass by several cytokines, such as macrophage chemotactic proteins secreted by cancer cells [4]. TAMs can also proliferate in the stroma of the tumor in response to stimulation from cancer cells [5]. On activation, TAMs can release a vast diversity of growth factors, proteolytic enzymes, cytokines, and inflammatory mediators, many of which are key agents in tumor progression, angiogenesis, and metastasis. Recently, TAMs were shown to be an important interface between tumor cells and the immune system, and may promote neoplastic growth and progression in several ways, including increasing cancer cell proliferation, enhancing angiogenesis, facilitating cancer cell invasion and metastasis, and suppressing human immune responses [6–8].

The association between TAM density (determined by average of macrophage counts under 400× field microscopy) and tumor proliferation, angiogenesis, and the clinical course and outcome of human cancer has been investigated in a number of studies. A high TAM density has been reported to correlate with a high proliferation index of cancer cells in breast cancer,

prostate cancer, and endometrial cancers [9–11]. TAM density was also reported to be associated with the differentiation of cancer cells and tumor size in a variety of human cancers, including breast cancer, bladder cancer, and glioma [12–14]. An association between macrophage infiltration and tumor-associated angiogenesis has also been demonstrated [14–20]. TAM density has been shown to correlate with tumor microvessel density (MVD) in a variety of human cancers, including endometrial, ovarian, breast, prostate, bladder, melanoma, and central nervous system malignancies [14–20]. Tsutsui *et al.* [21] showed that TAM density correlated with MVD and vascular endothelial cell growth factor (VEGF) protein expression in 249 patients with invasive ductal carcinoma. Takanami *et al.* [22] demonstrated that TAM infiltration was associated with MVD angiogenesis in pulmonary adenocarcinoma. Other studies showed that TAM density was associated with expression of angiogenesis factors, including interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), VEGF, and hypoxia inducible factor 2α (HIF- 2α) [23–26]. An association between TAMs and tumor invasiveness and metastasis has also been demonstrated in human cancer, including breast cancer: patients with a high TAM density show a trend to higher regional lymph node metastasis [21]. The association between TAM density and clinical outcome has been examined in several studies. Extensive TAM infiltration has been shown to correlate with a poor prognosis in a variety of human carcinomas, including breast, cervix, bladder, and prostate cancers and glioma [6, 10, 11, 13, 14]. Leek *et al.* [6] showed that a higher CD68-positive macrophage index was significantly associated with a worse prognosis for both relapse-free and overall survival in 101 breast cancer patients. Lee *et al.* [12] showed that in 75 breast cancer patients diffuse TAM infiltration was associated with a high tumor grade, tumor necrosis, and large tumor size, and indicated a poor prognosis. Furthermore, Volodko *et al.* [9] and Goede *et al.* [27] demonstrated that intensive TAM infiltration was strongly associated with a high tumor grade and poor prognosis in invasive ductal carcinoma of the breast. In prostate cancer, Lissbrant *et al.* [10] showed that TAM density correlated positively with tumor angiogenesis and a shorter survival time. Salvesen *et al.* [11] reported that in 60 cervical cancer patients both TAM density and tumor cell VEGF protein expression were significantly increased in patients with an aggressive tumor, and that a high average macrophage counts in the tumor correlated with high cancer cell proliferative activity and decreased survival. In addition, Fujimoto *et al.* [28] showed an association between TAM density, IL-8 protein expression, and microvessel count (MVC) in 80 cervical cancer patients, and that high level of IL-8 expression by TAMs was an indicator of poor prognosis. Hanada *et al.* [13] showed that, in 63 bladder transition cell carcinoma patients, a high TAM density correlated with tumor invasiveness, tumor stage, tumor grade, vascular

invasion, and distant metastasis, and was associated with an adverse prognosis. In lung cancer, Koukourakis *et al.* [17] showed that a high TAM density was linked to a poor prognosis in 141 non-small cell lung cancer (NSCLC) patients. To evaluate the prognostic role of TAM in lung cancer, Chen *et al.* [16] measured TAM density in 41 NSCLC tumor surgical specimens by immunohistochemical staining with anti-CD68 antibodies, and correlated TAM density with intratumoral MVC, tumoral IL-8 expression, and clinical outcome. They found that TAM density correlated significantly and positively with tumoral IL-8 expression and intratumoral MVC. A high TAM density was also associated with a short relapse-free survival. Consistent with this, Takanami *et al.* [22] showed that high TAM density was significantly associated with a high microvessel density and a poor prognosis in 113 lung adenocarcinoma patients. Other studies have shown that a high TAM density is an adverse prognostic indicator in uveal melanoma, prostate, endometrial, bladder, and lung cancer (by univariate or multivariate analysis) [10, 11, 13, 17, 29], although a few studies found no association between TAM density and clinical outcome in several human cancers, such as astrocytomas and ovary cancers [30, 31].

How TAMs contribute to an adverse prognosis in lung cancer has undergone extensive investigation, and these studies have identified several different possible mechanisms linking TAMs to tumor progression or invasion in human solid cancers (Fig. 1). First, several studies have demonstrated an association between increased tumor vascularity and macrophage infiltration in several human cancers [18–20], suggesting that TAMs enhance the angiogenic potential of tumors. Macrophage infiltration has been shown to correlate with vessel density in endometrial, ovarian, breast, and central nervous system malignancies [14–20]. The angiogenesis factors secreted by TAMs have been shown to be chemokines [IL-8, migration inhibitory factor (MIF), etc.], VEGF, TNF-α, thymidine phosphorylase, and a variety of cytokines, including granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), IL-1, IL-6, and prostanoids [32–34]. In addition, TAMs may enhance angiogenesis by increasing procoagulant activity and fibrin deposition [35]. On the other hand, studies have shown that a lack of macrophages may result in decreased angiogenesis and thereby cancer cell death [6, 36]. Second, TAMs have been reported to produce growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β), cytokines (IL-6, L-1, and TNF-α), and ornithine, which can enhance tumor cell replication and growth [34]. Third, TAMs can enhance cancer cell invasion and dissemination by increasing the production of cytokines (TNF-α, IL-1), extracellular matrix-degrading proteinases [such as matrix metalloproteinases (MMPs)], and plasminogen activator [37, 38].

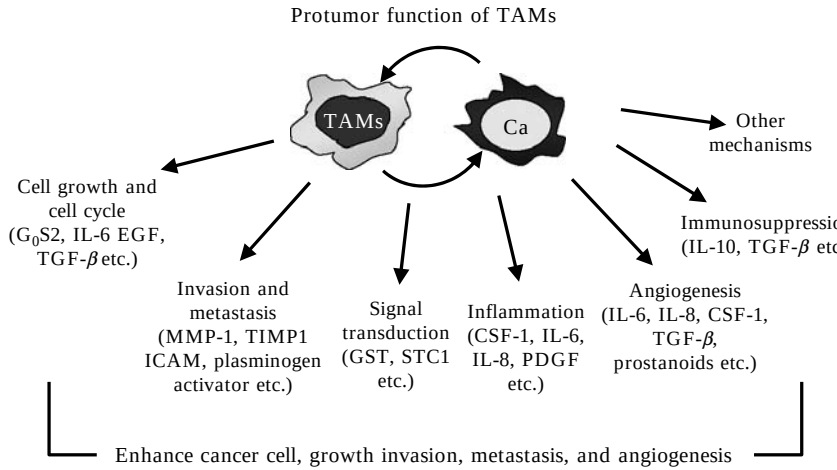

FIG. 1. A possible mechanism by which tumor-associated macrophages (TAMs) contribute to protumorigenesis effects and an adverse prognosis in cancer patients. After education by the tumor microenvironment, TAMs may express and secrete a variety of factors, which enhance cancer cell growth, invasion, metastasis, angiogenesis, signal transduction, and inflammation, and inflammation can stimulate tumorigenesis or metastasis. G_0S2, G_0/G_1 switch gene 2; EGF, epidermal growth factor; MMP-1, matrix metalloproteinase-1; TIMP-1, matrix metalloproteinase tissue inhibitor-1; ICAM, intracellular adhesion molecule; STC-1, stanniocalcin-1; CSF-1, colony stimulation factor-1; PDGF, platelet-derived growth factor [34, 49]. (This figure is modified from Fig. 1 in Ref. [49] with permission.)

For example, urokinase-type plasminogen activator (uPA) is a serine protease involved in extracellular matrix degradation. Upregulation of uPA expression has been reported in TAMs in several human cancers, and this can subsequently enhance tumor angiogenesis, invasion, and metastasis [39, 40]. Furthermore, uPA levels have been shown to correlate with reduced relapse-free and overall survival in breast cancer [41]. MMPs are a family of matrix-degrading enzymes, which includes collagenase (MMP-1), gelatinase A (MMP-2), stromelysin (MMP-3), matrilysin (MMP-7), gelatinase B (MMP-9), and other MMPs. TAMs can produce most forms of MMPs, and MMP-9 produced by TAMs was shown to correlate with the metastatic potential of a variety of human cancers [42–44]. MMP-9 can degrade basement membrane type IV collagen, elastin, gelatin, and other glycan core proteins, and can directly accomplish cleavage of galectin-3, transforming growth factor-β (TGF-β), and plasminogen, that is, activities that can substantially regulate tumor cell invasion and angiogenesis. Macrophage colony stimulating factor (M-CSF) is another agent by which TAMs can promote cancer cells invasiveness and metastasis. M-CSF can convert TAMs into mature functional

osteoclasts, which can increase bone resorption and facilitate bone metastasis of human cancers [45]. Fourth, TAMs can induce expression of inhibitory prostaglandin E2 (PGE2) and cytokines (IL-10), which suppress cell-mediated immune responses and inhibit cytotoxic T cell function and nature killer (NK) cell and lymphokine-activated killer cell cytotoxicity, and this can further promote tumor growth and expansion [46–48]. Other possible mechanisms contributing to the protumor function of TAMs are that TAMs may promote the epithelial–mesenchymal transition of cancer cells (our unpublished data showed that the TAMs can increase epithelial–mesenchymal transition-related genes expression in lung cancer cell after coculture). Finally, TAMs were also reported to be able to induce resistance of cancer cells to chemotherapeutic and hormone-therapeutic agents [49].

4. Antitumor Activity of TAMs and the Association Between High TAM Density and a Good Prognosis

The tumoricidal activity of activated macrophages has been studied for several decades. In contrast to the protumor effect of TAMs in human cancers, some investigations showed that TAMs may have important anti-tumor activity in human malignancy. Macrophages can become tumoricidal for cancer cells after they are activated by antibodies, cytokines [such as interferon-γ (IFN-γ)], or lipopolysaccharide (LPS). The tumoricidal effects of TAMs after activation include direct cytotoxicity against cancer cells and indirect cytotoxicity due to the secretion of products that can induce anti-tumor effects of other immune-related cells.

Macrophages form an important part of the immune cell or inflammatory cell infiltrate in tumors and are found in virtually all human malignancies. Although current evidence suggests that TAMs can be ''educated'' in the tumor microenvironment and subsequently reprogrammed to suppress host defense against cancer or to produce tumor growth-promoting factors, they have been shown to have tumoricidal effects in certain circumstances in some human cancers. As described above, most studies have shown that TAMs have protumorigenesis activity, may promote invasion activity, and were negatively associated with the patient survival in a variety of human cancers. However, some investigations showed that TAMs are associated with good patient prognosis in human cancers, including gastric and prostate cancers [15, 50]. Shimura *et al*. [15] showed that, in 85 prostate cancer patients, high TAM density correlated negatively with clinical stage and was associated with decreased lymph node metastasis and with a good prognosis. Migita *et al*. [50] showed that, in 104 gastric cancer patients, TAM density was higher in patients with intestinal-type disease rather than diffuse-type and

in those without liver metastasis than in those with liver metastasis, and suggested that TAM density correlated with a better clinical outcome. In lung cancer, Kerr *et al.* [51] showed that a high TAM density was associated with tumor regression and a favorable prognosis. Furthermore, Funada *et al.* [52] reported that peritumoral macrophage infiltration was associated with less lymph node metastasis and a good prognosis in colorectal cancer patients.

Welsh *et al.* [53] recently evaluated the relationship between tumor islet macrophages (TAMs that invade and infiltrate into the tumor nest, which may play a cytotoxic effect against cancer cell) and survival in 175 patients with surgically resected NSCLC. An increased tumor islet macrophage density or increased islet/stromal macrophage ratio was associated with longer survival in both early and advanced lung cancer groups. In contrast, increasing stromal macrophage density was an independent predictor of the reduced survival. The tumor islet macrophage density and tumor islet/stromal macrophage ratio were favorably independent prognostic indicators in patients with NSCLC. Islet TAM density had the greatest effect: 5-year survival was 52.9% in patients with an islet macrophage density greater than the median value of 131 cells/mm^2, but only 7.7% when the density was less than the median. Interestingly, patients with a high islet TAM density, but incomplete resection, survived markedly longer than patients with a low islet TAM density but complete resection. Similarly, Ohno *et al.* [54] showed that a high islet TAM density was associated with increased survival in gastric carcinoma patients. These results suggest that TAMs in different microanatomical areas or TAMs with different phenotypes in tumors may have different functions (protumor activity or antitumor activity). Stromal macrophages play a role in enhancing stromal formation and angiogenesis, which can subsequently promote tumor growth and metastasis [14–20]. On the other hand, tumor islet macrophages have cytotoxic effects and can inhibit tumor growth and spread [53, 54]. The tumor microenvironment can also modify TAM phenotype. Stromal TAMs express proteinase and proangiogenesis factors and promote tumor growth and metastasis in a variety of human cancers. In contrast, tumor islet TAMs express nitric oxide synthase and TNF-α, both of which have tumoricidal effects against prostate cancer [13]. Furthermore, islet TAMs can present tumor-associated antigens to cytotoxic T cells, which play an important role in the tumor cell destruction [54]. Macrophage infiltration was shown to correlate positively with CD8 T cell infiltration in gastric cancer [54].

The possible antitumor activity of TAMs may involve several mechanisms (Fig. 2). First, activated macrophages can kill cancer cells by direct cellular cytotoxicity, which involves direct contact between the macrophage and cancer cell and the translocation of lysosomal organelles from cytotoxic

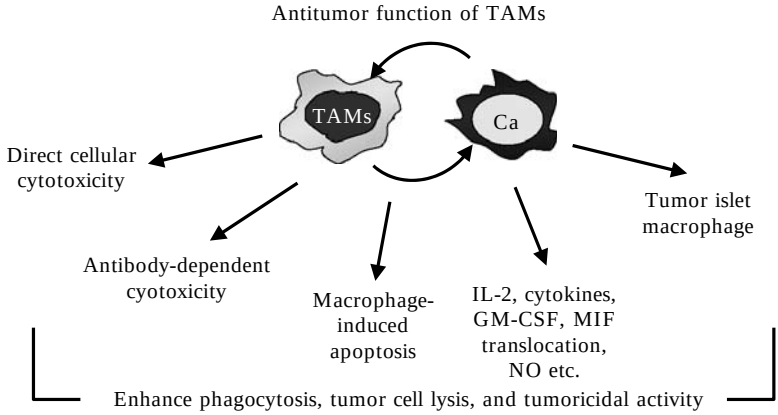

FIG. 2. Potential antitumor functions of tumor-associated macrophage (TAM) against cancer cells. After interaction with cancer cells, TAMs may enhance cancer cell phagocytosis, tumor cell lysis, and tumoricidal activity by direct cytotoxicity and antibody-dependent cytotoxicity, inducing apoptosis, expressing and secreting a variety of factors and cytokines, and other unknown mechanisms. The tumor islet macrophage is a cytotoxic phenotype TAM [49, 53]. (This figure is modified from Fig. 2 in Ref. [49] with permission.)

macrophages into the cytoplasm of cancer cells, with subsequent tumor lysis [34]. Second, macrophages can cause antibody-dependent cellular cytotoxicity by binding to antibody-coated cancer cells via their Fc receptor. TAMs have been shown to express CD16 (one type of Fc receptor) and have been suggested to play an important role in antibody-dependent tumor cytotoxicity [34, 55]. Third, many macrophage secretory products, such as ecosanoids (prostaglandins and leukotrienes), cytokine (IL-1, TNF-α), free radicals, NO, and enzymes (arginase), have cytotoxic or cytostatic activity against cancer cells [56–59]. Fourth, activated macrophages can induce apoptosis of cancer cells. This macrophage-induced apoptosis has been demonstrated in lymphoma, mastocytoma, and fibroblast cell lines [60–62]. Other factors may also contribute to the antitumor activity of TAMs, such as the expression of macrophage MIF, GM-CSF, and IL-2. Several studies have shown that MIF mRNA and protein levels are higher in tumors than in surrounding normal tissues and that cancer patients with tumor expression of MIF have a better prognosis than those lacking MIF expression [63–65]. Shinohara *et al.* [66] showed that transfection of the murine GM-CSF gene into human colon cancer cells increases their sensitivity to TAM-mediated tumor cell lysis *in vitro*. Incubation with GM-CSF induces CD11b expression on macrophages and increases the attachment of TAMs to cancer cells, thus increasing their tumoricidal activity [66]. IL-12 secreted by macrophages was also shown to be important for tumoricidal activity. Narvaiza *et al.* [67] showed

that cotransfection of IL-12 and IFN-γ-inducible protein-10 (IP-10) into murine colorectal adenocarcinoma cells resulted in total tumor eradication. Finally, although tumor islet macrophages have potential antitumor activity and have prognostic significance in NSCLC, the biologic explanation for their antitumor effect is still unclear. Further studies are needed to clarify the antitumor mechanism of tumor islet macrophages in NSCLC.

5. Polarization of TAM into M1 and M2 Phenotype Macrophage in Tumor Microenvironment

Recent evidence has demonstrated that polarization of TAMs into different phenotype subsets (M1 or M2) may play an important role on regulation of their function in tumor microenvironment. Cells belonging to the monocyte–macrophage lineage have long time been recognized to be heterogeneous, and macrophage heterogeneity is likely to reflect the plasticity and versatility of these cells in response to exposure to microenvironmental signals. Cytokines and microbial products profoundly and differentially affect the function of mononuclear phagocytes. Mononuclear phagocytes often function as control switches of the immune system, securing the balance between pro- and anti-inflammatory reactions. Depending on the activating stimuli, these cells can develop into different subsets: classically (M1) or alternatively (M2) activated mononuclear phagocytes (including M2a, M2b, and M2c), and the molecular and functional characterization of which is a current topic of investigation. Evidence has also shown that IFN-γ with LPS or TNF can polarize macrophage into M1 phenotype, which can induce Th1 response, type 1 inflammation, and involved in killing of pathogen or tumor cells. In contrast, IL-4, and IL-13, immunocomplex and toll-like receptor, and IL-10 had been shown to be able to polarize macrophage into M2a, M2b, and M2c phenotype, respectively, which involves in the Th2 response, allergy, killing of parasite, immunoregulation, matrix deposition, remodeling, and tumor promotion. The surface receptor, arginine metabolism, cytokine and chemokine secretion, and transcriptional program are different from each other (Fig. 3) [68].

In summary, TAMs play a complex and multifaceted role in the regulation of tumor growth and development. They have been shown to be directly tumoricidal and to stimulate the antitumor activity of other immune-related cells in several studies. However, there is accumulating evidence that cancer cells can modify the tumoricidal activity of TAMs and can sometimes even reprogram TAM activity in a protumorigenesis direction, that is, promote the growth, survival, and metastasis of the tumors themselves. There is substantial evidence that the interaction between TAMs and cancer cells can modify the extracellular

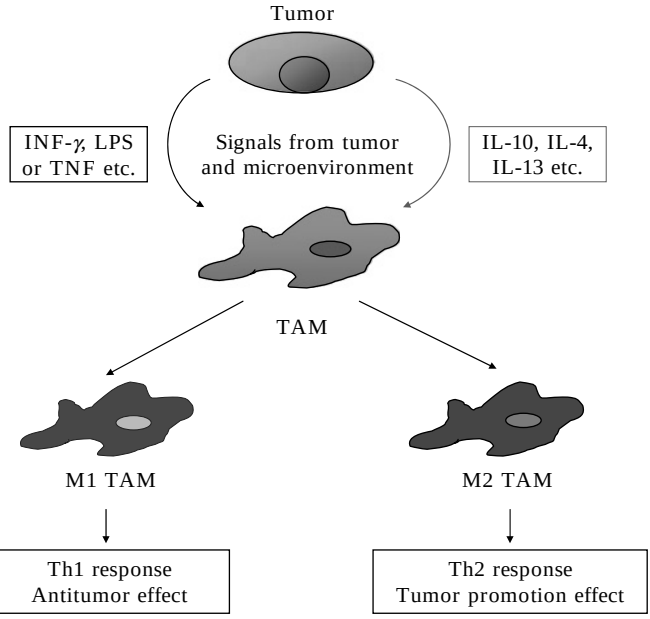

FIG. 3. Polarization of tumor-associated macrophage (TAM) into M1 or M2 phenotype macrophage by signals from tumor and the microenvironment. Cancer cell and tumor microenvironment can modify the TAMs function by producing different cytokines. IFN-γ, lipopolysaccharide (LPS), or tumor necrosis factor (TNF) can polarize TAM into M1 phenotype, and IL-10, IL-4, and IL-13 can polarize TAM into M2 phenotype macrophage. M1 phenotype macrophage can induce Th1 response and had antitumor effects, while M2 phenotype macrophage can induce Th 2 response which had tumor-promotion effects.

matrix and enhance cancer cell invasion and metastasis. Thus, TAMs may have either positive or negative effects on tumor growth, and their effects on tumor progression and invasion may depend on the tumor microenvironment, tumor-derived regulation, the differentiation of TAMs into cytotoxic macrophages, and other unclear molecular immunologic mechanisms. Further investigations are needed to elucidate the mechanism of regulation of TAM function in human malignancies, including lung cancer, and this may help in the future design of adjunctive therapy for human malignancy.

6. Effect of TAMs on Cancer Cells

It is becoming clear that inflammatory cells in the tumor microenvironment play an indispensable role in the cancer progression. This may explain why many cancers arise at sites of chronic irritation and inflammation [69].

Among these inflammatory cells, which include monocytes/macrophages, lymphocytes, neutrophils, and mast cells, the macrophage is the pivotal inflammatory cell within the tumor stroma. Recently, a number of reports have shown that TAMs constitute an important interface between tumor cells and the immune system, and that they might influence neoplastic growth and progression in several ways [6–8]. Tumor-infiltrating macrophages (TIMs) have also been shown to correlate with vessel density in ovarian [30] and breast [19] cancer and other malignancies [14], and have been associated with VEGF and epidermal growth factor receptor (EGFR) expression in cancer cells [70]. TIMs can be activated in malignant tumors and this may contribute to tumor angiogenesis [71, 72], and there is also a significant correlation between the number of infiltrating macrophages and angiogenesis [10, 28]. A high density of TAMs has been associated with angiogenesis and an adverse prognosis in lung adenocarcinoma [22].

Angiogenesis is required for tumor growth, progression, and metastasis [73, 74]. Several studies have demonstrated that a high intratumoral MVC correlates with tumor advancement, systemic metastasis, and prognosis in several human cancers, including melanoma, breast cancer, colon cancer, and lung cancer [75–78]. Angiogenesis is a complicated process that involves the degradation of the basement membrane and invasion of the stroma by endothelial cells, which then proliferate, migrate, and become organized into a capillary structure [79]. This process is regulated by the local activity of a variety of angiogenic factors, such as IL-8, VEGF, and basic fibroblast growth factor (bFGF) [74, 80, 81]. Our previous study [82] demonstrated that the presence of infiltrating macrophages in sections from lung cancer patients is accompanied by increased levels of IL-8 mRNA and positively correlated with tumor angiogenesis and negatively with patient survival.

The interaction between the tumor and surrounding stromal cells, such as macrophages, fibroblasts, and endothelial cells, is complex. In this chapter, we mainly focus on the interaction between cancer cells and TAMs. It has been shown that macrophages are attracted by monocyte chemotactic protein-1 and TGF-β1 secreted by tumor cells and that tumor production of TGF-β1 is responsible for activating macrophages [83]. On activation, TAMs release a vast diversity of growth factors, proteolytic enzymes, cytokines, and inflammatory mediators. Many of these are key agents in angiogenesis. White *et al.* [32] showed increased expression of angiogenic cysteine-amino acid-cysteine (CXC) chemokines, such as IL-8, in macrophages after coculture with NSCLC cells, indicating that cancer cells can stimulate inflammatory cells to express increased amounts of angiogenic factors. Using a human monocyte cell line model, THP-1, and an *in vitro* coculture apparatus

(Transwell), we also demonstrated that marked IL-8 mRNA expression (4.5-fold increase) is induced in macrophages after interaction with lung cancer cells [82]. TAM-derived angiogenic factors include VEGF, bFGF, platelet-derived endothelial cell growth factor, TNF-α, and IL-8 [70, 84].

However, TAMs are not the only source of increased angiogenic factors in the tumor microenvironment. The study of Liss *et al*. [83] showed that tumor cells themselves can be activated by macrophages and secrete angiogenic factors, which might contribute to tumor angiogenesis in head and neck squamous cell carcinomas. Our previous study [82] also revealed that IL-8 expression in cancer cells can be dramatically increased after interaction with macrophages. In this interaction, IL-8 expression was synergistically increased in both macrophages and cancer cells. Which cell is the predominant source of the angiogenic factors is still controversial. However, it is certain that TAMs play an important initiator role in the regulation of IL-8 expression in cancer cells. In addition, the amplification and propagation of IL-8 expression in cocultures is seen not only with the cancer cells mentioned above, but also with other tumor cell types (osteosarcoma and hepatoma) [82], and it has been suggested that increased IL-8 expression may be a common feature of the cancer cell/macrophage interaction.

7. Possible Regulatory Mechanism of Gene Expression in TAMs or Cancer Cells After the TAM–Cancer Cell Interaction

Paracrine regulation between cancer cells and TAMs has been observed and may play an important role in the tumor angiogenesis [82, 83]. Furthermore, using macrophage-cocultured cancer cells to sensitize naïve cancer cells, it was demonstrated that not only paracrine, but also autocrine regulation was seen in the lung cancer cells/TAMs cocultures [85]. This autocrine effect is also seen with different lung cancer cell lines [82], suggesting that autocrine regulation of IL-8 expression is a rather general phenomenon in the lung cancer cells. This suggests that autocrine regulation might play a crucial role in the tumor progression, particularly in cancer cells that have not interacted directly with TAMs but have been stimulated by TAM-activated cancer cells. It is well known that TNF-α activates angiogenic factors in several human tumor cell types and in vascular endothelial cells [84, 86], and a previous report hinted that TNF-α and IL1-α, produced by activated macrophages, are involved in tumor progression and angiogenesis in human malignant melanoma [84]. Moreover, TNF-α and IL1-α, secreted by activated monocytes and macrophages, enhance the production of IL-8 and VEGF in tumor cells *in vitro* [71, 83, 84, 87]. Our previous report [82] also

showed that protein levels of NF-κB, TNF-α, and IL1-α were elevated in both the TAMs and lung cancer cells after coculture and that IL-8 expression was still induced in cancer cells in the absence of macrophages by exogenously added recombinant human TNF-α and IL1-α. These findings show that TNF-α and IL1-α are involved in IL-8 mRNA induction in cancer cells/TAMs cocultures through autocrine and paracrine regulation. Using a reporter gene assay and electrophoretic mobility shift assay (EMSA), we also found that both NF-κB and AP-1 are involved in controlling IL-8 gene expression in lung cancer cells cocultured with TAMs. This multiple control of IL-8 gene expression in cocultures is consistent with the results of a previous study [88]. We can conclude that autocrine and paracrine effects on tumor angiogenesis are mediated, in part, through the NF-κB pathway and can be modulated by TNF-α and IL1-α.

As illustrated in Fig. 4, cancer cells attract macrophages that may produce inflammatory cytokines, such as TNF-α and IL1-α, which are then secreted into the surrounding environment. Increased TNF-α and IL1-α levels might induce lung cancer cells to activate NF-κB and subsequently increase IL-8 expression in cancer cells. Interestingly, lung cancer cells might regulate the production of IL-8 by themselves or by adjacent lung cancer cells, and induce more IL-8 production in an autocrine fashion, which would induce more angiogenesis in lung cancer.

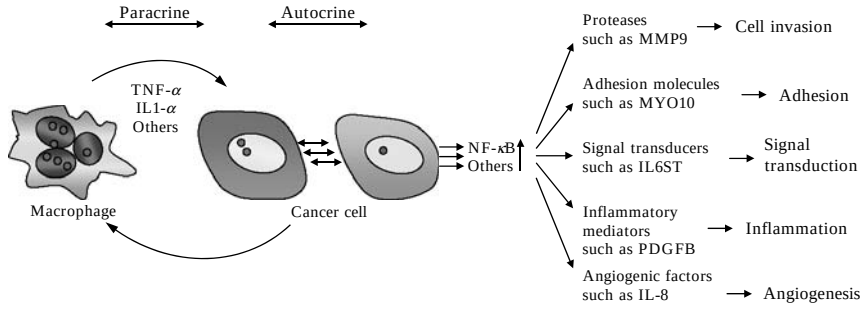

FIG. 4. Possible interactions between macrophages and cancer cells. Macrophages may first be attracted by chemokines derived from cancer cells, then the activated macrophages produce angiogenic factors, such as tumor necrosis factor-α (TNF-α) and interleukin1-α (IL1-α), and stimulate the cancer cells to produce more angiogenic factors and other factors. Meanwhile, stimulated cancer cells, by providing TNF-α, IL1-α, or other factors, may also stimulate neighboring cancer cells and macrophages to produce angiogenic factors and other factors. The autocrine and paracrine effects on cancer cells after interaction with macrophages are mediated partly through the NF-κB pathway, which may be involved in regulating cell invasion, adhesion, signal transduction, inflammation, and angiogenesis.

8. Effect of TAMs on Stromal Cells in the Tumor Microenvironment

In addition to cancer cells, TAMs interact with stromal cells, including fibroblast and endothelial cells, in the tumor microenvironment. It has been shown that fibroblasts play a prominent role in the tumor progression and metastasis [89]. Cocultures of fibroblasts and NSCLC cells show increased levels of IL-8 mRNA and protein in both the cancer cells and fibroblasts [88], demonstrating that cancer cells stimulate stromal cells to express larger amounts of angiogenic factors. However, little is known about the effect of TAMs on fibroblasts. Our previous study [85] demonstrated that both fibroblasts and bronchial epithelial cells sensitized by macrophages show a significant increase in IL-8 mRNA levels. However, in contrast to the results obtained from the cancer cell/TAMs cocultures, induction of IL-8 expression in normal lung fibroblasts and bronchial epithelial cells might be not through TNF-α or IL1-α. It is reasonable to speculate that other pathways regulate IL-8 induction in fibroblasts or bronchial epithelial cells. In terms of endothelial cells, as far as we are aware, no reports have been published about their interaction with TAMs. Nevertheless, our unpublished data show that several important angiogenic factors are induced in endothelial cells after interaction with TAMs.

9. A Global View on Changes in Gene Expression in Cancer Cells After Interaction with TAMs

To examine the global changes in protumorigenic gene expression in cancer cells after interaction with TAMs, microarray approach is a good choice [90]. Using this system, we are able to perform large-scale analyses of the genes involved in the interaction between cancer cells and TAMs, and this information should help in exploring the complex interactions between cancer cells and TAMs that orchestrate the process of tumor progression and metastasis. In our previous study, 50 genes showing significant up-regulation in cancer cell after their interaction with macrophage were identified using cDNA microarray [16]. Most of these changes had never been previously reported in cancer cell/macrophage cocultures. However, many of the genes involved had been previously reported to be associated with angiogenesis and metastasis, including IL-6 [91], IL-8 [82], MMP-9 and uPA receptor [92], stanniocalcin-1 (STC-1) [93], intracellular adhesion molecule-1 (ICAM-1) [94], and MMP-1 [95]. As mentioned above, the interaction between macrophages and cancer cells can upregulate IL-8 expression. This process is mediated, in part, through the NF-κB pathway [82, 85]. The microarray

analysis further supported the idea that IL-6, IL-8, M-CSF, MMPs, and ICAM-1 are involved in the NF-κB pathway [96, 97]. Because inflammatory cells may be involved in regulating the production of angiogenic factors by cancer cells, anti-inflammatory agents may have the potential to impede IL-8 induction and suppress angiogenesis initiated by inflammatory cells. Some nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have been shown to reduce the risk of developing colorectal and breast cancers [98, 99]. We have tested several anti-inflammatory drugs commonly used clinically in our previous studies and found that most inhibit the expression of angiogenic factor IL-8 [16, 82, 86]. Although their possible mechanisms of action are quite different [100–105], their inhibitory effect is, for the most part, finally mediated through the NF-κB pathway (Fig. 4). On the basis of the above results, TAMs might therefore be a potential target of chemotherapy using anti-inflammatory agents.

10. Implication of TAMs in Immunotherapy of Human Cancers

Since macrophages can infiltrate tumor tissue or have a tendency to be recruited to the tumor site, this suggests a new approach to cancer immunotherapy. Macrophages are known to have antitumor activity and are thought to have the potential to mediate tumor cytotoxicity and to stimulate antitumor effects of other immune-related cells. However, in the majority of human cancers, cancer cells can escape these macrophage-associated defense mechanisms. The possible mechanisms by which cancer cells escape macrophage-associated antitumor activity include [106–108]: (1) cancer cells do not express specific surface antigens that can be recognized by macrophages; (2) TAMs are modified by the tumor microenvironment, loose their cytotoxicity toward cancer cells, and are even redirected to protumorigenesis pathways; (3) after interaction with cancer cells, TAMs become suppressive for tumor-specific T- and NK-cell cytotoxicity.

Although there is evidence that the antitumor activity of TAMs can be suppressed and their protumorigenesis activity stimulated by the tumor microenvironment in the majority of human cancers, there is still a lot of potential to enhance antitumor activity by stimulation or activation of TAMs. A number of basic and clinical studies (including clinical trials) using activated macrophages in the immunotherapy of human cancers have been reported (reviewed in Ref. [109]).

One approach is to activate macrophages using biological response modifiers, such as muramyl tripeptide phosphatidylethanolamine (MTP-PE), GM-CSF, M-CSF, or IFN-γ, both *in vivo* (injection of patients with biological response modifiers) or *in vitro* (adoptive transfer of macrophages

treated with biological response modifiers). Asano *et al.* [110] showed that liposomal MTP-PE increased cytokine expression in monocytes and prolonged relapse-free survival time in osteosarcoma patients with lung metastasis in a Phase II clinical study. GM-CSF therapy in patients with lymphoma, breast cancer, or neuroblastoma was shown to increase antibody-dependent cytotoxicity and endogenous TNF-α levels [111], but produced no clinical response (regression of tumor) in Phase I and II studies [109]. In terms of adoptive cellular immunotherapy, although biological responses, including increases in cytokine levels, have been shown, clinical responses have been almost absent [112–114].

The second approach is to use gene transfer to induce and enhance the antitumor effect of TAMs. Recently, genetic modification of tumor cells with cytokines, adhesion molecules, or MHC molecules has resulted in activation of immune cells, induction of immune responses, and facilitation of cancer cell recognition and killing. Dranoff *et al.* [115] showed that transfection of the GM-CSF gene into murine melanoma cells initiated an effective and long-lasting anti-tumor response. Sanda *et al.* [116] also showed that vaccination with prostate cancer cells transfected with GM-CSF resulted in a significant increase in tumor-free survival of mice inoculated with the tumor. Morita *et al.* [117] showed that transfection of M-CSF into Lewis lung carcinoma cells prolonged the survival of mice injected with the transfected carcinoma cells compared to those injected with nontransfected cells and prevented lung metastasis. Dong *et al.* [118] showed that inoculation of mice with human prostate cancer cells transfected with IFN-β inhibited tumor growth and lymph node metastases. In animal studies, increased TAM infiltration of the tumor and inhibition of angiogenesis were found to correlate with GM-CSF production. In addition to these animal studies, several human clinical trials of gene therapy have been recently performed [119–122]. Treatment of patients with renal cell carcinoma with irradiated autologous GM-CSF transfected renal cell carcinoma cells resulted in a decrease in lung metastasis [120]. Vaccination with irradiated autologous melanoma cells transfected with the GM-CSF gene also resulted in extensive cancer cell destruction in melanoma patients [119]. Furthermore, using the tendency of TAMs to accumulate around the tumor nest, several investigators have started to transfect macrophages with genes encoding colony stimulation factor-1 (CSF-1), INF-γ, tumor antigens, antiangiogenic agents, and prodrug activation enzymes to enhance tumoricidal effects [123–126]. The results showed that these macrophages can effectively deliver gene therapy to the tumor mass but the clinical response remains to be determined. In conclusion, although a number of animal or human studies have demonstrated that gene therapy can enhance the tumoricidal effect of TAMs or other immune-related cells, further clinical trials are required to elucidate its effectiveness in human cancer.

11. Concluding Remarks

Macrophages can be recruited to tumors by a variety of cytokines secreted by tumor cells and become TAMs that can play a role in stimulating tumor growth and progression or, in contrast, in killing tumor cells and inhibiting metastasis. The exact role that TAMs play in the tumor depends on the state of macrophage activation, modification of macrophage function by the tumor microenvironment, the status of tumor development, the anatomic locus of macrophage infiltration, and the gene expression profile and phenotype of the TAMs. How TAMs are "educated" in the tumor microenvironment to show protumorigenesis or antitumor activity is a complex process and still under investigation. The interactions between TAMs and cancer cells and between TAMs and other stromal cells have recently been shown to be important in the modification of TAM function. The gene expression changes seen after interaction between cancer cells, TAMs, and other stromal cells may be involved in the regulation of the protumorigenesis or antitumor effects of TAM. The ability to direct TAMs toward antitumor effects may be helpful in the treatment of human cancer, and limited clinical studies have shown a potential effect of this immunotherapeutic treatment. Further studies to understand the mechanism of the modification of TAM function in the tumor are needed and should provide important information that may permit the development of more potent immunotherapies for human cancers.

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