

Neuro-endocrine-immune Crosstalk and Implications for Cancer Therapy

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Abbreviations: ACh: Acetylcholine; ACTH: Adrenocorticotrophic Hormone; ALC: Absolute Lymphocyte Count; AVP: Arginine Vasopressin; AR: Androgen Receptor; BM: Bone Marrow; CD40L: Cd40 Ligand; CNS: Central Nervous System; CRH: Corticotropin-Releasing Hormone; DC: Dendritic Cell; DP: Double Positive (Cd4+Cd8+) Cells; DTH: Delayed Type Hypersensitivity; HIV: Human Immunodeficiency Virus; HPA: Hypothalamic-Pituitary-Adrenal Axis; HSCT: Hematopoietic Stem Cell Transplant; ER α : Estrogen Receptor α ; ER β : Estrogen Receptor Beta; GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor; GnRH: Gonadotropin-Releasing Hormone; GVHD: Graft-Versus-Host Disease; IL: Interleukin; LHRH: Luteinizing Hormone-Releasing Hormone; LPS: Lipopolysaccharide; MS: Multiple Sclerosis - Norepinephrine; NK Cells: Natural Killer Cells; PVN: Paraventricular Nucleus; RA: Rheumatoid Arthritis; siRNA: Small Interfering RNA; TERT: Telomerase Reverse Transcriptase; TGF β : Transforming Growth Factor Beta; TLR: Toll-Like Receptor; TNF: Tumor Necrosis Factor; TREG: Regulatory T Cells

Introduction

Although the immune system has often been regarded as functioning independently in protecting the organism against foreign intruders, the last two or three decades have provided accumulating evidence that the nervous system, the endocrine, and the immune system are connected physiologically and act in a synchronized manner to mediate the body's quick and precise response to environmental stress [1]. While physically separated, these systems interact in a bidirectional way via a complex and tightly regulated network of neuropeptides, hormones, cytokines, and chemokines, which serve to mount a coordinated response to danger and maintain homeostasis [2]. These reciprocal interactions are dynamic, allowing for a constant crosstalk between the brain and the immune system, mainly through the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis [3,4]. Since the early evidence that inflammatory signals in the periphery can alter signaling in the hypothalamus [5], the focus has been on identifying a common molecular basis for this bidirectional communication shared through receptors and signaling ligands which cross sequestered anatomical locations [6]. Recent research suggests that neurotransmitters and neurohormones act by binding not only to their classical receptors on target cells in the nervous and endocrine systems, but also to receptors on various immune cells [7], thereby affecting many immunological processes in a powerful and rapid manner [8]. Primary and secondary lymphoid organs are innervated by nervous terminals secreting a variety of neurotransmitters [8]. Moreover, T-cells (and other immune cells) can also produce and secrete endogenous neuropeptides either spontaneously or after induction by external stimuli [8,9] which then act in an autocrine or paracrine manner to modulate pivotal immune functions, or cross anatomical barriers to facilitate the bidirectional crosstalk with other cells such as neurons or glia [8].

It is now well established that receptors for the parasympathetic mediator acetylcholine (ACh) and the sympathetic mediator norepinephrine (NE) are expressed on various immune cells such as macrophages, neutrophils, and lymphocytes [10-12]. For instance, activation of the ACh receptor on human macrophages exposed to bacterial lipopolysaccharide (LPS) significantly attenuates the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1 β , IL-6, and IL-18, but not the production of anti-inflammatory cytokines (such as IL-10) [13]. Similarly, direct stimulation of the peripheral vagus nerve during lethal endotoxemia in rats inhibits TNF synthesis in the liver, prevents the development of a shock-like response [13], and reduces paw swelling, and inhibits the development of acute arthritis after injection of the inflammatory chemical carrageenan. This blunting of an inflammatory response with vagal stimulation demonstrates that the cholinergic system functions as an anti-inflammatory mechanism in localized and systemic inflammation [14]. There is also evidence that the sympathetic neurotransmitter norepinephrine can also inhibit macrophage activation and suppress TNF synthesis with a resultant increase in IL-10 release early in the course of a systemic infection [15]. This may seem contrary to the classical teaching that the sympathetic and parasympathetic nervous systems function in opposite directions; in many situations the two systems function synergistically to limit the deleterious effects of a non-specific defense reaction.

Perhaps the quintessential reciprocal interaction between these systems in orchestrating the response to environmental stress is the modulation of the body's immune responses through the hypothalamic-pituitary-adrenal (HPA) [3,4] and hypothalamic-pituitary-gonadal axes. Studies as early as the 1980s demonstrated that cytokines play a pivotal role in this "chemical talk" by acting as immuno-neuroendocrine modulators [16]. Activated immune (e.g. monocytes, neutrophils, basophils, eosinophils, lymphocytes) and accessory cells (e.g. endothelial cells, fibroblasts, tissue macrophages) at the site of peripheral infection or inflammation secrete pro-inflammatory cytokines and other neuropeptide mediators of inflammation. How these signal transducing neuroendocrine stresses reach the CNS is still incompletely understood, but may involve special transport systems, direct activation of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) neurons in the median eminence

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(which is outside of the blood-brain barrier), or stimulation of the noradrenergic stress system [17]. The hypothalamic paraventricular nucleus (PVN) releases CRH and activates the anterior pituitary to produce adrenocorticotrophic hormone (ACTH), which activates the adrenals to secrete glucocorticoids (cortisol), which ultimately downregulate the immune response. HPA axis activation during inflammation acts as an important protective mechanism through endogenous cortisol release which in turn limits the immune reaction through inhibition of immune cell activation, suppression of cytokine production, suppression of the cyclooxygenase-2 pathway leading to decreased production of prostanoids, platelet-activating factor, and nitric oxide, and decreased expression of adhesion molecules and their receptors [17]. Glucocorticoids seem to preferentially suppress the function of type I helper (Th1) T lymphocytes and induce resistance to Th1 cytokines [17].

Gonadal steroid hormones regulate immune responses

More than 100 years have passed since Calzolari published his initial report of the positive effect of castration on thymus development in rabbits castrated before sexual maturity. However, until recently, little was known of the effects of gonadal steroid hormones on immunity. Of late, researchers have begun to place greater emphasis on the interaction of the immune and endocrine systems in the regulation of innate and acquired immune responses. The differential susceptibility to autoimmunity between sexes [18-20], or the changes in cell-mediated immunity seen during menstrual cycle and pregnancy [21-23] suggest that sex hormones may indeed modulate immune responses.

Androgens are generally viewed as negative regulators of immune function. The presence of the androgen receptor (AR) has been documented in lymphoid and nonlymphoid cells of thymus and bone marrow, but not in mature lymphocytes [24,25], suggesting that the major impact of androgens may be on the developmental maturation of T and B cells. Castration of male animals results in significant thymic enlargement and increase in thymus weight and thymocyte number [26-28], a phenomenon which is also observed in the setting of defective androgen action (the androgen-resistant testicular feminization mouse [29]). It has also been shown that androgen deprivation stimulates thymic T cell output and results in increased numbers of phenotypically naïve CD4+ and CD8+ peripheral T cells (approximately 2 weeks after castration) and enhances antigen-specific immune responses in postpubertal male mice [30]. In addition, androgen deprivation exerts a stimulatory impact on B-cell lymphopoiesis in the bone marrow (BM) [31,32], resulting in expansion of splenic and peripheral B-cell populations [33] and enhanced production of autoreactive antibodies [32]. Testosterone replacement in castrated mice, on the other hand, results in thymic regression and a significant decrease in thymocyte numbers, with a shift toward expression of mature thymocyte phenotypes, a decrease in double-positive (DP) phenotype (CD4+CD8+) T cells, and a relative predominance of the CD4-CD8+ suppressor/cytotoxic over the CD4+CD8- helper phenotype T cells [28]. Potential mechanisms include acceleration of thymocyte apoptosis [34], AR-mediated induction of downregulatory cytokines such as transforming growth factor beta (TGF- β) [35], or changes in thymocyte differentiation and maturation [36].

Initially, estrogen and progesterone's effects on the immune system appeared to be contradictory, until it was realized that the female sex steroid hormones have biphasic dose effects on immunity depending on physiological state, hormonal dose, and concentration [20]. For

instance, lower levels of estrogen seem to enhance, whereas higher levels (such as those in pregnancy) inhibit specific immune activities [20]. Serum progesterone and estrogen levels rise 5 to 10-fold during pregnancy [37]; these heightened hormonal levels are thought to help control the development and prevent rejection of the semi-allogeneic fetus by the maternal immune system [38,39]. This tolerogenic state is the result of the unique hormonal environment during pregnancy, which favors a Th2 response and, at the same time, halts the progression of Th1 immune responses associated with certain autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), or uveitis [20,40]. It is not entirely known, however, how exactly the dynamic hormonal changes during normal ovulatory cycling and pregnancy modulate the body's immune homeostasis and response to illnesses such as cancer.

Experimental studies imply that, on one hand, "low-dose" estrogen increases antibody production [41-43] and autoimmunity [44,45], while on the other hand, it decreases T-cell mediated delayed-type hypersensitivity (DTH) [41,46-48], suppresses leukocyte production in the bone marrow and granulocyte-mediated inflammation [49] as well as natural killer (NK)-cell mediated cytotoxicity [43,50,51]. Mammalian cells express 2 receptor isoforms for estrogen receptor (ER), ER α and ER β [52], although their relative contribution to estrogen-mediated changes in hematopoiesis is still under considerable debate [53]. Animal studies also suggest that ER subtypes have different roles in females and males [54]; for instance, ER α , but not ER β , seems to be necessary for full thymic development in male mice [54,55], whereas expression of ER β is required for estradiol-mediated thymic cortex atrophy and thymocyte phenotype shift in females [54]. Experimentally, estrogens have been shown to induce thymic atrophy and loss of lymphoid elements from the thymic cortex [56,57] at least partly, by increasing thymocyte apoptosis. In a study with ovariectomized female rats, ovariectomy produced a marked increase in thymus weight and had a profound effect on the thymocyte profile, leading to an increase in the CD4+CD8+ (DP) immature cells, with a relative decrease in the proportion of mature cells believed to harbor potentially autoreactive cell clones. This effect could be reversed by physiological doses of 17 β -estradiol [57]. Estrogen seems to rescue naive autoreactive B cells that normally are deleted and causes them to mature to a marginal zone phenotype and further leads to activation of this cell population [44,58]. Loss of estrogen through ovariectomy also upregulates B lymphopoiesis in the bone marrow and increases myeloid cell differentiation into the monocyte-macrophage lineage [59]. Estrogen receptor beta knockout (ER β -/-) mice develop pronounced splenomegaly that is much more severe in females than in males, with myelogenous hyperplasia in bone marrow, an increase in the number of granulocytes and B lymphocytes in the peripheral blood, lymphadenopathy, and infiltration of leukocytes in the liver and lung resembling human chronic myeloid leukemia with lymphoid blast crisis [60]. Conversely, pregnancy or administration of exogenous estrogen was shown to inhibit B lymphopoiesis [61-63]. While some studies indicate that ER β is the main negative regulator of hematopoietic progenitor cells [60,64], others suggest that this effect can be mediated through either ER α or ER β [58,65]. The decreased lymphopoiesis may reflect an intrinsic B-cell response to estrogen of early lymphoid progenitors [62,66] and/or indirect estrogen-dependent regulation of production and secretion of cytokines, such as IL-7, required for the development of early B lymphopoietic stages [58,66]. Furthermore, engagement of ER α , seems to be a trigger for autoimmunity and leads to breakdown in B-cell tolerance, with increased survival to immunocompetence of high-

affinity autoreactive B lymphocytes. In contrast, ER β engagement did not alter B-cell selection [58]. In addition, some hormonal effects on immunity and autoimmunity could be mediated through direct effects on dendritic cell (DC) function [40]. Estrogen and progesterone seem to exert different effects on DC differentiation and activation. Estrogen-dependent activation of ER α appears to be responsible for normal granulocyte-macrophage colony stimulating factor (GM-CSF) induced dendritic cell (DC) differentiation and acquisition of effector functions [67]. ER α -deficient DCs fail to upregulate MHC class II and CD86 molecules in response to microbial pathogens, which could account for their reduced capacity to prime naïve CD4+ T lymphocytes. Although they retain the ability to produce pro-inflammatory cytokines (e.g. IL-12, IL-6) upon toll-like receptor (TLR) engagement, ER α -deficient DCs are defective in their ability to secrete such cytokines in response to CD40-CD40L (CD40 ligand/CD154) interaction [67]. High-dose estrogen, on the other hand, induced IL-10 production by mouse splenic DCs [68] in one study, suggesting that this may be a mechanism by which high estrogen levels could facilitate expansion of tolerogenic DCs and explains the increase in the number and suppressive activity of regulatory T cells (Treg) associated with pregnancy and exogenous estrogen administration [38,69]. The Th2 dominance of pregnancy is further emphasized by the tolerogenic and immunosuppressive effects of progesterone, which has been shown to decrease gene expression and production of Th-1 cytokines [70], increase the production of anti-inflammatory cytokines (such as IL-10 and IL-13) [71], and increase the proportion of Treg during pregnancy [22,23].

Given this dynamic interaction between the endocrine system and immunity, a crucial question arises: could sex hormones or gonadotropin-releasing hormone (GnRH)-analogs be used therapeutically or as adjuvants in patients with cancer? Sex steroids and their agonists/antagonists are extensively used in contraception, hormone replacement therapy, as well as the treatment of breast and prostate cancer, but there is relatively little information regarding their effect on modulating the host immune response to other disease states. In autoimmune diseases, the defining event is loss of T cell tolerance to T-cell antigens, as opposed to the Th2-biased immune homeostasis and tumor-associated immunologic tolerance seen in advanced cancer. While still incompletely elucidated, understanding of the precise cellular and molecular mechanisms by which hormones alter these various immune functions is likely to play a crucial role in understanding gender differences in response not only to autoimmune diseases, but also chronic inflammatory conditions, human immunodeficiency virus (HIV) infection, cancer, bone marrow transplantation, or graft-versus-host disease (GVHD). For instance, gender has been shown to be an important and independent predictor of clinical outcome and survival in cutaneous melanoma, with premenopausal females (but not women older than 60 years) experiencing an improved prognosis [72,73]. The presence of sex steroid receptors in hematopoietic elements [24,58,66], as well as thymic and BM stromal cells [74-76] suggests that they may have a direct or indirect effect on immunity and hematopoiesis. The sexual dimorphism of the immune response could be modulated by differences in T cell receptor signaling, expression of activation molecules on T lymphocytes and antigen-presenting cells [77], transcription or translation of cytokine genes, or lymphocyte homing [20].

In the last few years, a significant body of research has accumulated regarding the immune modulatory effects of gonadal steroid hormones; however, the literature is often confusing and generates opposite conclusions. The main shortcomings involve the fact that most studies

are done on single sex animals making it difficult to accurately assess gender-specific immune responses; moreover, it is well known that oftentimes animal models fall short of reproducing human conditions. Therefore, direct comparison of males and females to determine specific gender differences in the innate and adaptive immune response (including individual Th1/Th2 balance) is crucial, as they may translate in a different susceptibility and immunologic response to chronic inflammatory stress. Our laboratory's work is focused on understanding the hormone-driven immune responses in human cancer and reproduction by studying the parallels of neuroendocrine immune regulation in women with and without cancer as they relate to menstrual cycle and pregnancy, identifying signals associated with inflammation versus tolerance induction in physiological and pathological states, and comparing to indicators of chemotherapeutic response in cancer.

Sex steroids and immune reconstitution following hematopoietic stem cell transplantation

Androgens have been used empirically in the treatment of bone marrow failure syndromes such as dyskeratosis congenita or Fanconi anemia since the 1960's, although their exact mechanism of action on hematopoietic recovery is not completely understood. Recent preclinical data suggests that one potential explanation is that androgens may act by restoring telomerase expression in hematopoietic cells. Exposure of normal human bone-marrow derived CD34+ cells and of peripheral blood lymphocytes from patients heterozygous for telomerase mutations to androgens increased telomerase activity in one study [78]. This effect was abolished by letrozole, an aromatase inhibitor, but not by flutamide, an androgen receptor antagonist, suggesting that stimulation of telomerase activity by androgens is regulated mainly by aromatization [78]. Estradiol had a similar effect on *TERT* gene expression and telomerase enzymatic activity, which was abolished by tamoxifen and by down-regulation by small interfering RNA (siRNA) of estrogen receptor- α (ER α), but not ER β [78]. Estrogens have also been implicated in regulating cell proliferation fates by reprogramming the sizes of telomeres in normal reproductive tissues [79] and ovarian cancer cell lines [80].

In addition, the potential immune modulatory properties of currently used synthetic hormones and their agonists/antagonists could be investigated and utilized in conditions such as HIV, bone marrow transplantation, or after high-dose chemotherapy in cancer patients, when prompt immune reconstitution is essential [81]. Impaired lymphoid reconstitution following autologous hematopoietic stem cell transplant (HSCT) is known to increase opportunistic infections post transplant and to affect disease relapse and survival in multiple hematologic malignancies due to a presumptive lack of graft-versus-tumor effect [82-84]. Day 15 absolute lymphocyte count (ALC-15) after autologous HSCT has been reported to be a significant predictor of clinical outcome [82], with natural killer (NK) cells identified as a key lymphocyte subset affecting survival after transplant [82]. Post-autologous HSCT, granulocytic recovery is usually accomplished in 2 to 3 weeks, while recovery of lymphoid cell immunity seems to display a greater variability [85]. NK cells generally reach pretransplant, or at least normal, levels by 10-14 days, CD3+ and CD8+ T cells need about 3 months, while recovery of humoral immune response takes 3 to 6 months [85-87]. In contrast, total CD4+ T cells and certain subsets such as CD45RA+ T cells (predominantly naïve CD4 T cells) are depressed for more than 1 year post-transplant [86,88].

Similar to lymphocyte recovery as pivotal for survival post-autologous HSCT, several reports have also shown that early posttransplant lymphocyte recovery has a direct impact on survival in patients undergoing allogeneic HSCT [89-91] (and manuscript under review), indicating that this phenomenon may be a universal surrogate of immunologic reconstitution post-HSCT. However, recovery of immunity post-allogeneic HSCT is even more delayed and is often incomplete as compared to autologous HSCT due to the requirement of several months of immunosuppression and the potential development of GVHD [92]. Therefore, identification of new ways to modulate lymphoid and NK cell recovery after autologous and allogeneic HSCT could provide new immunomodulation strategies to reduce relapse, avoid infectious complications, and correct altered immune homeostasis.

In the last decade, there has been increasing interest on the potential effect of sex steroids on post-transplant immune reconstitution. For instance, androgen deprivation has been shown to shorten skin allograft rejection time in male mice [93]. Indeed, the suppressive influence of sex steroids on the thymus is well documented, with age-related thymic atrophy becoming more pronounced at the onset of puberty [94]. Although the loss of thymic function with age does not have immediate clinical consequences, it results in detrimental changes in the peripheral T cell pool, with a decreased export of naïve T cells and a compensatory increase in the memory T cell population [95,96]. Therefore, the age-related thymic involution resulting in defective thymopoiesis becomes important in situations when prompt regeneration of the peripheral T cell pool is needed following destruction, such as administration of cytotoxic chemotherapy or stem cell transplantation [96]. Surgical or chemical (via luteinizing hormone-releasing hormone (LHRH) analogues) castration has been shown to reverse thymus atrophy in both males and females [26,97,98], while re-administration of synthetic sex steroids inhibits thymic growth and regeneration [98]. Moreover, androgen deprivation has been shown to accelerate restoration of lymphocyte levels within the thymus and periphery of castrated mice treated with cytotoxic (lymphopenic) chemotherapy and after autologous and allogeneic stem cell transplant without leading to an increase in graft-versus-host disease [87,99-101], thereby providing new strategies for post-transplant immune reconstitution. The increased thymic cellularity involved all of the thymocyte subsets and early T lineage progenitors; in addition, surgical castration seems to induce early repair of damaged thymic stromal microenvironment resulting in enhanced production of chemokines and growth factors important for thymopoiesis [99]. Importantly, these observations have also been demonstrated in the clinical HSCT setting, where treatment with goserelin (an LHRH agonist) prior to HSCT resulting in improved thymopoiesis and disease-free survival without an increase in GVHD post-HSCT [102]. These results are encouraging and support further investigation into modulation of thymic function and immune responses using endocrine-based therapies.

Neuroendocrine-immune biorhythms in malignant diseases

Rhythmic phenomena are typical for all levels of biological organization, with periods ranging from centuries and decades (evolution of species and ecological systems), to milliseconds of electrical potential in nerve and cardiac cells [103]. There are natural fluctuations in the levels of gonadotropin and sex hormones during the menstrual cycle and even with diurnal rhythm. Similarly, in humans, immune cells of either innate (e.g., neutrophils, monocytes, natural killer [NK]-cells) or adaptive immune system (lymphocytes [T- and

B-cells]) have been shown to exhibit circadian variations [104]. Under regular sleep-wake conditions leukocytes show robust diurnal rhythms which seem to be regulated by the body's two major stress hormones, cortisol and epinephrine, with peak counts at night or during the day, depending on the cell type [4]. Most recently, Lee and colleagues have demonstrated similar fluctuations in the peripheral blood T cells and NK cells, as well as NK cytotoxicity during menstrual cycle in healthy women volunteers [105].

Circadian immune changes driven by biological rhythms have been shown to cause disease-associated diurnal changes in rheumatological disorders [106,107] which correspond to the diurnal variations in plasma glucocorticoid concentration. Biological rhythms with a periodicity longer than 24 hours have also been detected in experimental models of inflammation [107]. Muir et al. [108] described a circaseptan rhythm of paw edema with peak inflammation occurring every 6-7 days after paraffin injection in rats. Circannual variations were also identified with greater inflammatory symptoms in the spring compared to fall and winter [109].

Temporal variations in the immune response to malignant disease have been underemphasized but they are likely to have significant implications for the pathogenesis and treatment of these diseases. For instance, carefully designed observational studies have shown that people whose circadian rhythms are chronically disrupted are more prone to developing cancer (e.g. night-shift workers and breast cancer risk) [110,111]. Likewise, cancer growth may disturb biorhythms in the host [112,113]. We have recently demonstrated that similar dynamics control the immune response to advanced cancer and that the immune infradian biorhythms in patients with metastatic melanoma extend beyond the 24-hour sleep-wake variability [4] seen in normal immune homeostatic states (Dronca et. al, in press). Moreover, our data also suggests that these immune biorhythms may be therapeutically relevant with respect to timing of chemotherapy administration and can have a dramatic impact on therapeutic outcome. Future studies will need to determine how normal immune and anti-tumor responses change longitudinally during menstrual cycle and pregnancy; correlating the levels and rhythms of sex steroids and cytokines with the body's response to disease and disease severity will also be important.

Conclusion

Although gender differences in autoimmune diseases are well recognized, the sexual dimorphism in the immune response and the importance of sex hormones in promoting differences between men and women in the susceptibility and immunological responsiveness to other chronic conditions, such as cancer, need further study. In the last few decades, there has been an explosion of information on the role of hormones in certain malignancies and also on the immune mechanisms important in the pathogenesis of these diseases. Important questions remain regarding specific gender differences in the innate and adaptive immune response to acute and chronic stress and the mechanism underlying differential neuroendocrine immune regulation of specific disease states. Expanding our understanding of the dynamic intercommunication between the nervous, endocrine, and immune systems during remodeling of the immune response in pathological conditions such as cancer, bone marrow transplantation, and with administration of chemotherapy, will help identify new therapeutic targets and immunomodulatory strategies and provide insight into personalized cancer treatment.

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