

Review Article

Gender differences in urothelial bladder cancer. Effects of natural killer lymphocyte immunity

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Abstract: Men are more likely to develop cancer than women. In fact, male predominance is one of the most consistent cancer epidemiology findings. Additionally, men have a poorer prognosis and an increased risk of secondary malignancies compared to women. These differences have been investigated in order to better understand cancer and to better treat both men and women. In this review, we discuss factors that may cause this gender difference, focusing on urothelial bladder cancer (UBC) pathogenesis. We consider physiological factors that may cause higher male cancer rates, including differences in X chromosome gene expression. We discuss how androgens may promote bladder cancer development directly by stimulating bladder urothelium and indirectly by suppressing immunity. We are particularly interested in natural killer (NK) cells because they are important, but often overlooked anti-cancer lymphocytes.

Key words: Urothelial bladder cancer, Natural Killer Cells, Androgens, Immunosuppression, X Chromosome

1. Introduction

The Gender Divide: “Instead of ignoring our differences, we need to accept and transcend them.” Sheryl Sandberg [1]

UBC is common, with a 2.4% lifetime risk [2]. UBC is much more common in men: out of the estimated 83,750 new UBC cases in America in 2021, about 64,280 are in men and 19,450 are in women [3]. UBC also is more deadly in men: in 2021 it is estimated to kill 12,260 men and 4,940 women [3]. Even when controlling for increased male exposure to carcinogens, such as tobacco and industrial chemicals, men are at an increased risk [4]. Gender differences in cancer outcomes can be due to biological or social factors. For example, well-known variances in occupation, workplace exposures, and social networks could all influence cancer incidence and survival, but not be directly due to biological sex. In this review, we focus on biological sex differences that may affect UBC incidence and mortality. As we discuss below, observed UBC gender differences could be related to how androgens and the androgen receptor (AR) affect UBC development and progression. UBC gender disparity also could result if androgens suppress anti-cancer immunity. Moreover, greater transcription of some X chromosome genes may protect females from UBC.

Most UBC are identified early and are transurethraly resected. Adjuvant treatment often includes intravesicular infusion of cytotoxic chemotherapy or of Bacillus Calmette-Guerin (BCG) immunotherapy. While superficial tumors often respond to these treatments, UBC has a lifetime recurrence risk of 50-70% and requires long-term monitoring. As a result, UBC treatment and monitoring cost more than for any other cancer [5, 6]. It should be emphasized that UBC 5-year mortality rates have remained fairly consistent over the last 3 decades, despite advancements in treatment [2]. To help current and future patients, we must further study UBC pathogenesis. This review focuses on proposed biological reasons for high male incidence and mortality. What we learn from gender differences may allow us to better understand and treat UBC in both sexes.

2. The androgen receptor in bladder cancer

Encoded on the X chromosome, the androgen receptor (AR) binds to testosterone and related hormones. Testosterone stimulates cells through multiple mechanisms, classical and non-classical [7]. In the classical mechanism, the hormone passively diffuses into the cell and directly binds cytoplasmic AR. Increasing potency, testosterone is reduced by 5- α reductases in the

cytoplasm to dihydrotestosterone (DHT), which binds to AR with high affinity. Hormone binding changes AR conformation, which allows hormone-AR complexes to enter the nucleus and either enhance or depress transcription of multiple genes [7]. In addition, AR interacts with important cytoplasmic signaling molecules, including PI3K, Src, and Ras to initiate MAP kinase signaling [8]. In one non-classical mechanism, ZIP9 (SLC29A9) was identified as a non-AR cell surface androgen receptor. Upon testosterone binding, ZIP9 increased intracellular zinc and imparted an apoptotic signal [9, 10]. A surface androgen receptor that does not react with anti-AR antibody was detected at the surface of human T lymphocytes and androgen binding appeared to impart a calcium signal [11-13]. In those studies, T cell intracellular calcium was detected with Fura-2. However, Fura-2 has a 100-fold higher affinity for zinc than for calcium [14], so it is possible that ZIP9 is the T lymphocyte cell surface androgen receptor. Finally, inflammatory cytokines activate AR signalling in the absence of androgens [15, 16]. IL-18 activates AR via ERK, Src, and FAK signaling [15], while IL-6-activated STAT3 directly phosphorylates AR [16]. AR is widely expressed in both sexes, despite large differences in androgen levels. Both sexes express AR in the bladder, including urothelium, muscularis propria (detrusor muscle), and bladder neurons [17-19]. In castrated male rats, androgen replacement therapy significantly improved urothelial thickness and muscle fiber quantity, indicating an androgen effect on these structures [20].

High overall male cancer risk naturally suggested that AR and testosterone accelerate cancer initiation, progression, or metastasis. For prostate cancer (PCa), this relationship is well established. In the early 1940s, Charles Huggins castrated men to treat their PCa [21, 22]. The success of this and other hormonal therapies suggested that androgens are a driving force. Some prostate cell lines undergo apoptosis when androgen is withdrawn [23]. Androgen deprivation therapy (ADT) remains a PCa treatment mainstay, especially in advanced disease [24]. These treatments typically are effective early, but are not curative; PCa progress a median of 24 months after ADT initiation [25]. Increasing evidence suggests that even if PCa reaches the castration-resistant phase (growth in the presence of low, but detectable testosterone levels), AR nonetheless stimulates the cancer cells and continuing ADT is currently recommended [24].

In addition to PCa, many cancers are influenced by androgens. For example, castration of male rats reduced both chemical-induced pancreatic tumor burden and renal cell carcinoma; testosterone administration at least partly reversed this effect [26]. However, particularly

aggressive cancers often do not respond to ADT. Castrate-resistant PCa is a classic example. It most often arises after ADT, which selects for highly aggressive hormone-resistant cancer cells that survive chemotherapy and proliferate. Recent work has suggested a mechanism by which this may occur: Schroeder et al [27] found that in mice, the AR antagonist, flutamide, caused PCa cells to convert to a cancer stem cell (CSC) phenotype. The process was mediated through STAT3 signaling induced by IL-6. As noted above, STAT3 directly phosphorylates AR [16]. Blocking mouse IL-6 mice significantly reduced PCa stem cell numbers [27].

As a general principle, hormone-resistant cancers may arise from hormone-sensitive tissue. Breast cancers often are treated with tamoxifen, a selective estrogen receptor modulator that inhibits estrogen actions in breast tissue. However, some breast cancers resist hormone-based therapy. Many molecular mechanisms allow breast cancers to lose estrogen sensitivity [28]. MEL-18 overexpression was linked to resistance to tamoxifen therapy [28, 29]. Lee et al found that loss of MEL-18, a polycomb group protein, allowed breast cancer cells to decrease estrogen dependence [29]. Breast cancers that lose estrogen sensitivity typically have a more aggressive course and poorly respond to chemotherapy [30]. A notable example is triple-negative breast cancer (negative for ERBB2 (Her2/neu), estrogen receptor, and progesterone receptor protein expression), which is typically an aggressive tumor that does not respond to targeted chemotherapy agents [30]. Interestingly, triple-negative breast cancers of women often express AR and might respond to ADT or AR blockade [30]. Yet, triple-negative breast cancers that lose AR expression often have characteristics of primitive basal-like cancers with poor clinical course [31]. This suggests that AR signaling may help induce new breast cancers, but “dedifferentiated” primitive cancers may downregulate AR expression because they are driven by hormone-independent mechanisms. Similar phenomena have been observed in other cancers that become independent of the signaling pathways that characterize cells from the same tissue of origin.

Hormone independence sometimes arises early in tumor progression. Breast ductal in situ carcinomas likely are precursors of invasive carcinomas. A few of these non-invasive tumors are “triple-negative” and express other markers characteristic of highly aggressive invasive breast cancers [32, 33]. This evidence suggests that steroid hormone receptors and other important drivers of breast tissue growth can be lost early in the evolution of a few tumors. Analogously, most chronic myeloid leukemias respond well to imatinib and other protein tyrosine kinase inhibitors [34]. However, some leukemias fail treatment from the beginning, suggesting de novo

resistance [35]. Chronic myeloid leukemia de novo protein tyrosine kinase inhibitor resistance has been linked to elevated expression of the *PRKCH* gene, which encodes a protein kinase C signaling protein family member [36]. Interestingly, *PRKCH* gene expression is also high in chronic myeloid leukemia stem cells [36]. Even if hormone receptor signaling helps initiate new cancers, hormone independence may arise in cancer progression. Resistance to drugs that affect hormone stimulation and signature signaling pathways fits into Hanahan and Weinberg's carcinogenesis and cancer progression paradigm [37]. Tumors are characterized by increasing independence from the growth factors that normally control cell proliferation in their tissue of origin. Independence from particular growth signals may occur early, as noted above. Due to their high mutation rate, other cancers unleash a repertoire of new growth mechanisms and acquire growth signal independence later in tumor evolution [37].

3. UBC as an endocrine tumor

Growing evidence indicates that UBC, like PCa, is driven by androgens and AR signaling. Distinct from most epithelial tissues, urothelium is derived from the urogenital sinus, which also gives rise to prostate in males [38]. Therefore, it may be expected that prostate and urothelium share properties, such as AR-dependence. Many human UBC cell lines and 44-78% of UBC tumors express AR in both men and women [39, 40]. Moreover, stromal cells express AR in about half of UBC tumors [39]. The *AR* gene is polymorphic for exon 1 CAG repeat number, which encodes a tract of glutamine amino acid residues [41]. CAG repeat number inversely correlates with *AR* transcription and AR signal strength [41]. This suggests that the CAG-encoded polyglutamine tract interferes with *AR* transcription and possibly with AR protein function. Men with low *AR* CAG repeats are more likely to develop locally invasive or metastatic PCa [42]. Similarly, male UBC patients had fewer *AR* CAG repeats than control males, suggesting that AR signaling also may drive UBC [43].

In addition to spontaneously-arising cancers, carcinogen-driven UBC in mice were accelerated by AR and androgens. The carcinogen, BBN (N-butyl-N-(4-hydroxybutyl)nitrosamine), more quickly induced UBC in male mice than in female mice. BBN-induced male UBC was reduced by surgical or medical ADT and an AR-blocking drug [44] or by knockout of the *AR* gene [45]. Interestingly, *AR* knockout protected both male and female mice from BBN exposure, eliminating UBC incidence and greatly reducing bladder hyperplasia [45]. Finasteride (a 5 α -reductase inhibitor that prevents the conversion of testosterone to the

more potent androgen, DHT), was marginally protective [44]. In female mice, androgen treatment increased bladder tumor incidence. The significant ADT effect and the marginal protection offered by finasteride suggest an AR signaling threshold. 5 α -reductase inhibitors prevent conversion of testosterone to the more active DHT, but are associated with higher testosterone levels [46]. Therefore, mouse UBC might be driven equally by physiological male androgen signaling and by moderate androgen signaling found in the presence of 5 α -reductase inhibitors. This parallels findings in human PCa—men with the lowest 10% of testosterone levels were relatively protected from PCa, but men with the highest testosterone levels had no increased risk compared to men with moderate levels [47]. Furthermore, testosterone replacement therapy does not increase PCa risk [48]. If there is an androgen threshold in UBC, we would speculate that the relatively low androgen levels in women are insufficient to drive cancer. And even if low (female) androgen levels were sufficient to drive UBC development or progression, it is nonetheless possible that high androgens in males impart a greater UBC risk. In summary, a growing body of evidence shows how AR and androgen signaling promote UBC development and progression. Based on this evidence, we suggest that targeting AR will reduce UBC recurrence and progression.

Transcriptional coactivator proteins help mediate AR-dependent gene transcription and support AR protein stability [49]. The steroid hormone receptor coactivator, NCOA3 (AIB1), was overexpressed in about a third of human UBC, which was an independent predictor of poor progression-free survival in multivariate regression analysis [50]. Furthermore, *NCOA3* knockdown slowed human tumor xenograft growth in mice and *NCOA3* enforced expression increased tumor growth [50]. SiRNA knockdown of coactivator RNA in AR-positive UBC cell lines decreased androgen-induced proliferation [40]. AR coactivators NCOA1, NCOA2, NCOA3, CREBBP, and EP300 were expressed in 86-100% of UBC, even though AR was present in a minority of UBC [40, 44]. The presence of these coactivators when AR was absent suggests that they enhance non-AR-directed transcription in UBC cells.

We do not completely understand how androgens and AR control UBC. As in prostate cancer [49], some data suggest that UBC may initially present with androgen dependence but progressive UBC may lose this dependency. Izumi et al [51] reported that AR, estrogen receptor- α (ER α), and ER β nuclear staining was more frequent in normal appearing urothelium than in UBC. In men receiving ADT, AR-negative UBC went on to recur in 12 of 28 patients (43%), but

AR-positive UBC recurred in only 11 of 44 patients (23%). In multivariate analysis of men receiving ADT [51], only 2 factors were significant, UBC AR expression and UBC tumor number (single vs. multiple). This suggests that ADT delays or prevents UBC recurrence in AR-positive tumors. AR expression level in normal urothelium did not correlate with tumor recurrence and tumor cell ER α and ER β expression did not predict recurrence. UM-UC-3 UBC cells selected for AR expression had higher clonogenic growth and greater migration than parental UM-UC-3 cells after androgen treatment in vitro, suggesting that AR drives UBC growth and metastasis [52]. In another study, Izumi et al [53] studied UBC recurrences in patients who also were treated for PCa. UBC recurrence was less frequent in the ADT-treated PCa group (5-year actuarial recurrence-free survival: 76% v 40%; $P < 0.001$). In those men with UBC recurrence, ADT was associated with fewer recurrence episodes (5-year cumulative recurrence: 0.44 v 1.54; $P < 0.001$). Most patient characteristics would have been predicted to disfavor the ADT group (worse PCa disease, older age), but brachytherapy was higher in the non-ADT group and radiation is a UBC risk factor [54]. In addition, a greater percentage of the ADT group received BCG infusions [53], which prevent UBC recurrence [55-57]. Although intriguing, conclusions are weakened because the study was retrospective and multicenter [53]. Shiota et al [58] followed 228 men for recurrence of bladder cancer for an average of 3.6 years; 32 of 196 men received ADT or dutasteride (a 5 α -reductase inhibitor) for concomitant PCa. UBC recurred in 4 men with ADT or dutasteride (12.5%) and 59 men without (30.1%). Progression to muscle invasive cancer occurred in none of the men on ADT or dutasteride and in 6 men who were not on these therapies (3.1%). ADT (or dutasteride) was associated with significantly decreased risk of intravesical recurrence (hazard = 0.4, $p=0.024$). Although sample size was small and follow-up time was short, this study suggests that ADT or dutasteride may prevent UBC recurrence in high risk patients.

Based on 13 years follow-up of the Prostate, Lung, Colorectal, and Ovarian (PLCO) database, men who were treated with finasteride (a 5 α -reductase inhibitor) at any time during the survey had lower UBC incidence (hazard = 0.634), when also controlling for age and smoking [59]. The retrospective study was limited because finasteride dose and some risk factors (such as alcohol consumption) were not known. In two retrospective insurance record reviews in Taiwan, investigators found that 5 α -reductase inhibitors did [60] or did not [61] reduce the risk of UBC recurrence. One of the studies found that 5 α -reductase inhibitors prevented UBC deaths [61].

Interestingly, amplification of the gene that encodes 5 α -reductase was found in 12% of UBC queried in the TCGA database [60]. Interpretation of these studies is difficult because of the expected lower complications of urinary tract infection and hematuria in 5 α -reductase inhibitor users may have led to fewer exploratory cystoscopies and therefore fewer incidental UBC diagnoses. This may bias UBC discovery to the non-5 α -reductase inhibitor group. Despite the number of manuscripts showing that ADT and 5 α -reductase inhibitors reduces UBC incidence or recurrence, it should be noted that most of these studies are complicated by methodological flaws [62].

The most definitive study was performed in a double-blind prospective manner as part of the MTOPS trial [46]. Subjects were monitored for compliance, something that was impossible with retrospective studies. As expected, serum DHT levels were lower and testosterone levels were higher among the subjects treated with 5 α -reductase inhibitor drug [46]. UBC incidence was not statistically different in men with and without 5 α -reductase inhibitor treatment. We conclude that the literature on 5 α -reductase inhibitor treatment is inconsistent, but a prospective double-blind study failed to find protection against UBC incidence. Literature inconsistency correlates with weak findings in experimental animals [44]. One possible interpretation of these results is that there is a threshold effect of androgen signaling in promoting UBC--the moderate amount of androgen signaling retained in 5 α -reductase inhibitor users may be sufficient to promote UBC, whereas the lower level of androgen signaling under ADT may reduce UBC risk.

Investigators have shown a correlation between AR expression and increased transformation of normal bladder urothelium to UBC. Hsu et al [63] found that bladder-specific *AR* knockout mice developed fewer and less aggressive BBN chemical-induced UBC. They also showed that AR acted through a p53-mediated pathway, allowing more bladder cancer cells to survive apoptosis [63]. This suggests that AR signaling increases tumorigenesis and tumor cell survival. Luna-Velez et al reported that AR expression was significantly lower in muscle invasive disease than in nonmuscle invasive UBC; AR expression was the lowest in the most advanced T3 and T4 disease, compared with T1 UBC [52]. On the other hand, Mir et al [64] did not find a significant AR-related difference in time to death or rate of recurrence in a study of almost 500 patients with UBC muscle invasive tumors. Among the patients studied, 12.9% of the tumors expressed AR and there was no significant sex difference. AR likely affects UBC in complex ways that depend on cancer stage. AR signaling seems to initiate more UBCs, but we

do not know whether AR signaling causes more aggressive disease. Given that many tumors grow increasingly independent of hormones and signaling pathways during cancer progression [37], it would not be surprising that androgens drive early UBCs, but not late UBCs.

In a separate aspect of AR signaling, Miamoto's group [65] showed that androgens increased resistance to cisplatin-based chemotherapy of AR-expressing UBC cell lines. In a complementary fashion, AR blocking drugs increased cisplatin sensitivity [65, 66]. Among the mechanisms responsible, Miamoto's group [67] found that androgens reduced expression of an RNA- and ribosome-processing protein, BRIX1 (BXDC2) in UBC cell lines. Furthermore, AR and BRIX1 showed complementary expression patterns in UBC tissue sections [67]. Cisplatin-resistant cell lines had increased BRIX1 expression [67], but BRIX1 mechanisms in UBC are not clear. Although treatment of cell lines in vitro cannot be directly translated to the clinic, these studies suggest that AR antagonists may act synergistically with cytotoxic chemotherapy in UBC patients. As mentioned above, intravesicular BCG treatment commonly follows superficial UBC resection. BCG vaccination has many nonspecific effects, including nonspecific induction of "trained" immunity and lower levels of inflammatory serum proteins [68]. It is notable that the reduction of inflammatory proteins was much more pronounced in men than in women and correlated with pre-vaccine testosterone levels in males [68]. Preclinical data showed that BCG was more effective when AR was downregulated or absent, or in the presence of AR antagonist drugs [69, 70]. The mechanism is possibly related to the Rab27b vesicle protein, which may allow UBC to exocytose previously ingested BCG [69]. In a retrospective analysis of human biopsy samples, both AR and Rab27b protein expression correlated with higher UBC recurrence rates [69].

UBC incidence and the total number of UBC-related deaths are much higher in males than in females [3], but women who develop advanced, muscle-invasive bladder cancer fare worse than men [71]. As an exception to that general finding, women may have a better cancer specific mortality than men to neoadjuvant chemotherapy plus radical cystectomy [72]. This conclusion, which was based on two small studies [72], requires confirmation. Consistent with overall worse outcome for women, multivariate analysis suggested that female sex was a risk factor for recurrence after BCG therapy [73]. de Jong and colleagues [74] found that, compared with men, women were more likely to have the aggressive basal/squamous subtype, whereas men were more likely to have luminal papillary and neuroendocrine-like subtypes. It may be

significant that advanced UBC in men had higher androgen response activity across all luminal subtypes, compared to advanced UBC in women [74]. These findings support the concept that androgens help initiate UBC. However, some UBC progress to a more aggressive form, becoming androgen-independent through AR loss or other means. One molecular consequence of AR loss in advanced UBC is that AR suppresses transcription of *CD44* [75], which encodes a cancer stem cell marker that mediates UBC aggression [76]. AR mRNA negatively correlated with CD44 mRNA in UBC, both before and after chemotherapy [75]. Because of lower androgen levels in women, it should not be surprising that the smaller numbers of UBC female patients are enriched for more aggressive androgen-independent cancers. In contrast to androgens, the role of estrogens in UBC is less clear. Hsu et al [77, 78] reported that female mice lacking ER β were *less* susceptible to carcinogen-induced UBC, whereas female mice lacking ER α were *more* susceptible. These apparently discordant results suggest that there are distinct roles for ER α and ER β homodimers, in addition to ER α β heterodimers.

4. Androgenic Immune Suppression

Gender-based immune disparity is well-documented [79-81]. Men suffer more infectious disease complications [82, 83] and cancers [3, 84-86] than do women, including at elderly ages. Women generally respond better to vaccines, although exceptions exist [79, 87-91]. Autoimmune disease can be attributed to excess immunity and women are more susceptible to the most common autoimmune diseases [90]. Sex differences are observed in children as well. Infant boys are significantly more susceptible to infections than infant girls, a difference which is attributed to the androgen surge that boys experience at birth [90, 92-94]. In addition to androgens, male-female immune response differences might be linked to estrogens, progesterones, X linked genes, and socioeconomic factors. In the current pandemic, male gender has been found to be a strong risk factor for COVID-19 disease and death, whether or not overall SARS-CoV-2 infection rate is higher in men than women [95, 96]. Notably, the large overlap in risk factors and pathogenesis suggests mechanistic parallels in COVID-19 and PCa pathogenesis [96].

Early hematopoietic precursor cells express AR RNA in mouse bone marrow and in human bone marrow and cord blood [97]. However, some differentiated hematopoietic cells lose AR expression [97]. Using immunohistochemistry, Mantalaris and colleagues [98] detected AR protein in several bone marrow elements, including stromal cells, endothelial cells, M ϕ and other myeloid cells (although not eosinophils). AR expression was affected neither by sex nor by age

(range 1-92 years). However, Mantalaris et al [98] did not detect AR protein in bone marrow lymphocytes.

5. Androgens and Innate Immunity—Myeloid Cells

Myeloid cells are pivotal in anti-cancer immunity [37, 99-101]. Depending on the local cytokine and cellular microenvironment, myeloid cells promote tumor angiogenesis and growth, and suppress immunity. Alternatively, myeloid cells present tumor antigens to T cells and cooperate with NK cells to eliminate cancer cells [37, 99-109]. Developing myelocytes respond to androgens, but specific myeloid subsets become androgen independent at distinct differentiation stages. The common myeloid progenitor is stated to express AR RNA [26]. The common dendritic cell and the granulocyte-macrophage progenitor do not express AR, but mature myeloid cells do: macrophages (M ϕ), monocytes, neutrophils (both band cells and segmented), and mature mast cells [26, 98].

Among the myeloid cells, M ϕ are particularly important because they regulate tissue homeostasis [110-112]. M ϕ engulf apoptotic bodies, capture antigens and present them to T cells, and produce IL-1, IL-23, TNF- α , IFN β , IL-15, and other cytokines that shape immune responses [102, 108, 113-115]. The immediate precursors of tissue macrophages are blood monocytes. Myeloid-specific *AR* gene deletion reduces monocyte precursors in the mouse bone marrow [116]. *AR* knockout did not reduce total blood monocyte, but did reduce the proportion of classical monocytes [116], which are thought to enter tumor and inflammatory sites and develop into macrophages or dendritic cells. AR-blocking enzalutamide had similar effects in mouse bone marrow cells [116]. Gonadectomy elevates pro-inflammatory responses by increasing expression of toll like receptor 4 (TLR4) by male murine M ϕ [117]. TLR4 potently activates NF- κ B, a master proinflammatory transcription factor [118]. Some results, however, suggest that testosterone promotes specific proinflammatory cytokines in certain contexts. LPS-induced mouse M ϕ TNF- α production was elevated by testosterone and depressed by AR blockade and *AR* gene deletion [119, 120]. In contrast, testosterone did not affect LPS-stimulated TNF- α protein secretion by mouse fetal liver Kupffer cells, which are macrophage lineage [121]. However, high dose testosterone was used in the latter study [121], which might have caused secondary effects. Oxidized lipoprotein-stimulated human M ϕ made less TNF- α and IL-1 β in the presence of testosterone, but IL-6 expression did not change [122]. In that study, the M ϕ were derived from the monocytes of 50-70 year old human subjects [122]. Thus, testosterone and AR

signaling may differentially affect M ϕ depending upon the M ϕ source, the type of stimulus, and the cytokine secreted. Once activated by LPS, NF- κ B migrates to the nucleus and stimulates cytokine gene transcription and the cellular response to stress [118]. M ϕ NF- κ B expression and signaling are suppressed by AR activation, but not by estrogen receptor activation [123, 124]. Female M ϕ have higher TLR4 expression and increased phagocytosis and enhanced oxidative burst. Gonadectomy augments TLR4 expression in male mouse M ϕ , which increases the inflammatory response [117]. M ϕ involved in wound healing express AR. Wound healing is slower in males than in females, but healing was accelerated by an AR antagonist, flutamide, and by myeloid-specific *AR* gene deletion [119, 120]. Importantly, wound healing correlated negatively with testosterone levels in elderly men [119].

Neutrophils, the most abundant blood leukocyte, respond rapidly to bacterial infections and many cancers [125]. Evidence suggests that neutrophil differentiation from myeloid progenitors is directly promoted by androgens [126]. AR-deficient mice and androgen insensitive mice show profound neutropenia [126]. Following treatment with stanozolol, a testosterone analog, female mice increased neutrophil maturation rate [127]. Similarly, PCa patients treated with AR blockers may display neutropenia [128]. While it has been shown that low testosterone level reduces neutrophil numbers, we do not fully understand how AR signaling affects neutrophil function [129]. Some authors have shown that androgens suppress neutrophil production of proinflammatory cytokines and instead promote IL-10 production [130]. These data suggests that androgens are needed for neutrophil differentiation, either due to a lack of development or lack of survival, but that androgens dampen neutrophil inflammatory actions.

Dendritic cells (DCs) present antigens to T cells and also affect NK cell function [103, 104, 131]. DCs help connect NK cells to the adaptive immune system, and vice versa [105-107, 132]. DC transfer MHCII molecules to NK cells, but “MHCII-dressed” NK cells inhibit CD4 T cells [133]. DCs synthesize IL-15 and present it on their cell surface to stimulate NK cells to mature, divide, and survive [104, 134-136]. DC stimulate NK cells to become cytotoxic, including via granzyme B synthesis [104, 134, 136, 137]. Only a few studies have addressed possible direct effects of AR and androgens on DCs, but in general they seem to depress DC immune function [26]. This is controversial because some investigators have shown that DCs do not express AR [138]. DCs isolated from male mice after brain LCMV infection were less activated than in female mice [139]. Male sex reduced expression of DC MHCII and CD86

[139], These molecules respectively present antigen at the cell surface to CD4 T cells [140] and costimulate T cell CD28, which promotes T cell differentiation and survival [141]. Castration increased expression of MHCII, CD86, and other co-stimulatory molecules [142].

6. Androgens and Innate Immunity—NK Cells

NK cells help control cancer and kill both virus infected cells and neoplasms without antigen specificity or prior immunization [143-145]. NK cells attack tumors, including isolated cancer cells and metastases [109, 143-149]. Comprising 5 - 15% of blood lymphocytes in healthy people, NK lymphocytes are defined by CD56, CD16, or NKp46 (CD335) expression in the absence of the CD3 T cell receptor [143-145]. NK cell activation is regulated by multiple receptors [143-145]. Cell surface MHC class I molecules (MHCI, termed HLA class I in humans) present antigenic peptides to CD8 cytolytic T lymphocytes, which then divide, secrete IFN- γ and other cytokines, and kill antigen⁺ cells [140, 150]. In addition to stimulating CD8 T cells, MHCI molecules strongly modulate NK cell responses by engaging NK cell KIR, NKG2A, and LILRB1 inhibitory receptors [143, 145, 151, 152]. The contribution of MHCI recognition has been analyzed by blocking with anti-MHCI antibody. Blocking MHCI recognition with antibody permits NK cell attack on cells that express high levels of MHCI, but does not further increase attack on tumor cells that express little or no MHCI [153]. NK cells also express many stimulatory receptors [143-145]. For example, NKG2D binds to stress-activated ligands that are preferentially expressed on tumor cells and virus infected cells [143, 145, 147, 154]. When confronting stress-activated ligand-positive tumor cells, antibody blockade of NKG2D prevents robust NK cell activation [154].

AR may affect NK cell development. Acyline-mediated chemical castration (ADT) for two weeks increased circulating NK cells in men [155]. Testosterone replacement prevented these changes. After 4 weeks of recovery from ADT, NK cell number returned to normal levels; CXCR1 and NKG2D expression did not significantly change [155]. These findings indicate that androgens control human NK cell number. In the elderly, the immature CD56^{bright} to mature CD56^{dim} NK cell ratio was significantly higher in women than in men [156]. Female CD56^{dim} NK cells had higher cytotoxic granule exocytosis in response to K562 tumor cells and higher IFN- γ made in response to cross linking of NKp46. Plasma IL-15, a cytokine required for NK cell development and survival, did not differ by gender [156]. Therefore, although the mechanisms of the gender differences were not identified, the results suggest that testosterone

suppresses NK cell activity in elderly humans. We did not detect AR mRNA either CD56^{bright} or CD56^{dim} peripheral blood NK cells (data not shown); therefore, testosterone effects on mature NK cells likely are indirect. We further investigated whether androgens affected NK cell activity in the context of cancer treatment. After obtaining informed consent, 5 men were studied before and after ADT for metastatic PCa. The study was IRB-approved and consistent with the Helsinki Declaration. Peripheral blood was removed by venipuncture and the ability of NK cells in a mononuclear cell preparation to produce a representative cytokine (IFN- γ) and chemokine (MIP-1 β) using various forms of stimulation was compared pre and post ADT. We found that in response to IL-2, IL-15, or IL-12/IL18 stimulation, mature CD56^{dim} NK cell tended to make higher amounts of IFN- γ post-ADT, although the differences were only significant for the IL-2 stimulus (data not shown). However, both IL-15 and IL-12/IL-18 stimulated significantly higher MIP-1 β responses post-ADT, with the IL-2-stimulated responses trending higher (Figure 1). We also tested NK cell cytotoxic granule exocytosis (CD107a expression) and IFN- γ and MIP-1 β production stimulated by K562 leukemia cells or immobilized antibody to the NKp46 activating receptor. We found no significant differences or even trends in NK cell responses to those stimuli, comparing pre- and post-ADT. This shows that NK cells from men receiving ADT were not globally activated, but selectively responded to IL-2, IL-15, and IL-12/IL-18 cytokine stimuli. Although many changes take place with ADT initiation, including a reduction of PCa mass, our results are consistent with the hypothesis that androgens suppress NK cell IFN- γ and MIP-1 β responses to cytokines and that ADT relieves the suppression.

7. Androgens and Adaptive Immunity–B cells

Gender immune differences include how androgens affect lymphocyte development. Male mice with the inactivating Tfm *AR* mutation had increased B cell precursor number and increased mature B cells [157, 158]. In male mice, ADT and castration increased B cell lymphopoiesis in the bone marrow [159, 160]. Androgen treatment returned B cell levels to normal. Experiments with chimeric mice which express *AR* exclusively on either stromal cells or lymphoid cells in the bone marrow showed that stromal cell *AR* is essential to inhibit the B cell lymphopoiesis typically observed with androgen treatment [159]. IL-7-stimulated B cell precursors cultured independently of stromal cells did not respond to androgens [159]. This suggests that the B cell response to androgens is mediated by stromal cells. In contrast to these findings, B cell specific *AR* knockouts had elevated B cell lymphopoiesis, suggesting that mouse

B cell precursors are direct androgen targets. The effect of the general *AR* knockout was more pronounced than the B cell specific *AR* knockout, suggesting that both B cell and stromal *AR* suppress B cell development [159]. Further studies showed that DHT causes stromal cells to produce transforming growth factor- β , an anti-inflammatory cytokine that dampens immune responses. Relevant to this discussion, transforming growth factor- β suppresses IL-7 production, a cytokine that is required for B cell proliferation and differentiation [161].

B cells both activate and carry out adaptive immunity. First, B cells stimulate a special class of CD4 T follicular helper cells in the germinal center [162]. Later in the immune response, B cells develop into plasma cells that secrete antibody, which protects the host by several mechanisms [163]. In general, women produce relatively more antibody after vaccine [79, 88]. Low testosterone levels in men predict more B cells, and higher response to vaccines and to infection [88, 90]. Research suggests that mature B cells do not express AR; therefore these effects may be exerted by other leukocytes or on B cell precursors, when the AR is expressed [160, 164, 165].

8. Androgens and Adaptive Immunity–T cells

While research suggests that the androgenic effect on B cells is limited to development and stromal cell response, T cell response to androgens is more sustained and direct [166]. AR is highly expressed in lymphoid precursor cells and in supporting cells: marrow stromal cells and thymic epithelial cells [167]. It was established more than a century ago that castration causes thymic hypertrophy, which suggests that androgens regulate T cell development [80, 168-171]. Testosterone induced thymic involution [167, 170], while testosterone ablation causes the thymus to regrow [167]. Although AR has been detected in thymocytes, experiments with chimeric mice showed that thymic involution required AR in stromal cells, but not in bone-marrow derived T lymphocyte precursors: Olsen et al [167, 170] created chimeras differing in whether functional AR was expressed solely in thymocytes, hematopoietic progenitor cells in the thymus, or thymic epithelium. They found that thymic epithelial cell expression of AR was necessary for androgen responsiveness. In a key molecular step, androgens reduce thymic epithelial expression of δ -like 4, a Notch ligand that is required for T cell maturation [80, 172].

Mature T cells appear to express both classic cytoplasmic AR and a plasma membrane androgen receptor [11-13, 165, 173-175]. Exogenous DHT treatment skews mouse T cell activation, proliferation, and differentiation [8]. These effects are likely due to a combination of

intrinsic T cell effects [173] and to depression of antigen presenting cell MHC and costimulatory molecule expression or cytokine production [117, 119, 120, 122, 139, 142]. Androgens inhibit T cell-dependent antibody production [26, 88, 90]. Thus, androgens affect both lymphocyte development and mature T lymphocytes.

The study of androgen regulation is complicated by the many classes of T cells, each with unique function. CD4 T-helper 1 (Th1) cells are proinflammatory and produce cytokines that stimulate cell-mediated and innate responses, which help prevent tumors, and clear intracellular bacteria and viruses [176]. Kissick et al [173] showed that testosterone reduced CD4 Th1 differentiation by upregulating protein tyrosine phosphatase, non-receptor type 1 (Ptpn1), which negatively regulates many cellular processes. T cells expressed less Ptpn1 in PCa patients undergoing ADT than in control PCa patients [173] and (Haydn T. Kissick, personal communication 12 April 2019). CD4 T-helper 2 (Th2) cells control humoral immunity and the clearance of extracellular pathogens [176]. As with Th1 cells, androgens diminish Th2 responses. In parasitic infections, male mice had a suppressed Th2 response, which was associated with myeloid cell IL-18 upregulation. Thus normal male mice did not clear infection, but infection was cleared by castrated males, normal females, and ovariectomized females [177]. Th2 cells also quell inflammatory immune responses by secreting antiinflammatory cytokines [176, 178]. Experimental autoimmune encephalomyelitis is a commonly used experimental model for multiple sclerosis, a demyelinating autoimmune disease. In experimental autoimmune encephalomyelitis cell culture and mouse models, androgen treatment increased IL-10 production and decreased demyelinating disease in female mice [179]. The underlying mechanisms are not known, but IL-10 generally seems to protect against autoimmune disorders [178]. A c-Kit genetic defect had opposite effects on female and male SJL mice [180]. Female *KIT* defective mice had *reduced* experimental autoimmune encephalomyelitis severity, while male knockout mice had *increased* disease severity [180]. Increased severity in males was associated with reduced IL-4 and IL-10 Th2-like T cell responses and increased Th17 responses [180]. Importantly, increased disease severity in male *KIT* deficient mice correlated with decreased numbers of Innate Lymphoid Cell type 2 (ILC-2) cells [180]. Although androgens act directly on T cells [165], some effects are indirect. Androgens stimulated mast cells, which activated Innate Lymphoid Cell type 2 (ILC-2) cells, which in turn activated a Th2-like response [181]. Interestingly, testosterone, Fc receptor cross-linking, and *Mycobacterium tuberculosis* all

induce IL-33 production in male, but not in female, bone marrow-derived mast cells [181]. In specific inflammatory diseases, IL-33-driven Th2 responses ameliorate disease pathology [182].

Regulatory T cells (T_{reg}) modulate the immune response by reducing inflammation and by modulating response to self-antigens [183, 184]. Regulatory T cells serves as brakes on a variety of cells, including NK cells [185, 186]. Cancer patients often have high circulating T_{reg} levels, especially of naïve T_{reg} [187]. In the tumor environment, the ratio of regulatory to effector T cells correlates with poor outcome, but the absolute number of T_{reg} cells may or may not indicate worse prognosis, depending on the cancer type [188-193]. When Treg cells are depleted, mice better clear tumors [183, 185]. In culture, mouse and human T_{regs} dampen NK cell cytotoxicity against tumor cell lines [183, 194]. Androgens increase T_{reg} cells in vivo and in vitro. Waleki et al showed that this modulation was related to androgen-dependant acetylation of histone H4 at the *FOXP3* locus, a gene that is needed for T_{reg} cells to differentiate in the thymus and to function in the peripheral tissue [175, 195]. Androgen modulation of FoxP3 expression is one of many mechanisms by which androgens suppress immunity.

9. Chromosomal effects on UBC

In healthy females, each cell randomly silences one X chromosome at an early stage of embryogenesis [196]. However, X chromosome silencing is incomplete and about 15-25% of these genes are transcribed at least 10% of the level on the active X, and a few at nearly 100% [197, 198]. Some genes always escape complete inactivation, and some vary between XX females. Escape from complete silencing also differs by tissue and stage of development and increases with entry into the cell cycle [199, 200]. Within a given tissue compartment, escape from X chromosome inactivation appears stochastic. Single cell RNA sequencing reveals that most blood human plasmacytoid dendritic cells expressed a single allele of all 4 tested X chromosome loci, but some cells had biallelic expression by 1 to 4 of those loci [201]. Thus, compared to males, females express significantly more mRNA from certain X-linked genes. As discussed below, recent studies have shown that the X chromosome protects females, accounting for some of the gender disparity in UBC incidence. UBC and other solid tumor incidence are elevated in patients with Turner syndrome, which is characterized by XO, a single sex chromosome [202, 203]. Turner syndrome patients often produce less estrogen and less testosterone than typical XX women, due to hypoplastic ovaries. Therefore, elevated cancer incidence may be due to reduced X chromosome gene dosage or due to lower amounts of

immune-modifying estrogen. In contrast, solid tumor incidence is decreased in Klinefelter syndrome patients, who carry at least 2 X chromosomes and 1 Y chromosome, have small testicle size, and reduced testosterone [202]. As in females, X chromosome inactivation is incomplete on supernumerary X chromosomes in Klinefelter males [204]. As above, sex hormones, X chromosome number, or both might reduce cancer risk. Testosterone replacement in Klinefelter syndrome patients decreased serum immunoglobulin and decreased both B and T circulating cell numbers, identifying low testosterone level as largely responsible for Klinefelter effects on lymphocyte numbers [205]. To separate biological sex and associated hormone expression from sex chromosome composition, investigators have created “four core genotype” mice [206]. These animals have the sex-determining *SRY* gene deleted on the Y chromosome and inserted into an autosome, allowing biological sex (and sex hormones) to be separated from X and Y chromosome composition. Using “four core genotype” mice, Smith-Bouvier and colleagues showed that, regardless of biological sex and sex hormone levels, SJL mice with XX chromosomes were more susceptible to experimental autoimmune encephalitis than were SJL mice with XY chromosomes [207]. Two X chromosomes also conferred greater susceptibility to pristane-induced systemic lupus, regardless of biological sex or hormone status [207]. Incomplete X chromosome inactivation also protects from infection. Plasmacytoid dendritic cells and B cells from female subjects with biallelic TLR7 expression was associated with higher levels of type I interferons (IFN- α and IFN- β) and class switching to IgG, respectively [201, 204]. This is important for immune protection, because type I interferons are essential for early response to viral infection [208-210] and IgG clears pathogens through multiple mechanisms [211]. Escape from complete X chromosome inactivation by the CXCR3-encoding chemokine gene in T cells is linked to superior resistance to experimental *Leishmania* infection in female mice [212]. Aging changes immune cell gene expression in both sexes but unequally; men and women rapidly diverge in the 7th decade [213]. Because sex hormone differences are smaller in the elderly than in young adults, these observations suggest that some of the biggest gender-related immune differences are not due to the immediate sex hormone milieu. These and other data indicate that sex hormones and sex chromosomes independently modulate immunity.

Cancer risk has been studied in “four core genotype” mice. Kaneko and Li [214] used the BBN carcinogen to induce UBC. XY male mice were most susceptible, followed in order by XX males, XY females, and XX females. Survival was significantly different in each group. Thus,

both biological sex and sex chromosome composition contributed to UBC risk in mice. Investigating mechanism, Kaneko and Li [214] showed that the X-linked lysine demethylase 6A (*KDM6A*) gene is expressed more highly in the urothelium of XX males and females, than in the urothelium of XY males and females [214]. *KDM6A* gene deletion reduced expression of *Cdkn1a* and *Perp*, which are targets of the p53 tumor suppressor. Conditional *KDM6A* gene deletion caused upregulation of aging-associated genes and an aging phenotype in the mouse hematopoietic system [215]. Urothelium-specific *KDM6A* knockout increased carcinogen-induced UBC risk in female mice. Additional research showed that the mouse findings are relevant to human disease. UBC from female patients had more *KDM6A* expression than did UBC from male patients [214]. Furthermore, low tumor *KDM6A* expression was associated with higher UBC tumor stage in women, but not in men. *KDM6A* mutations and low mRNA levels correlated with poor disease-free survival in women, but not in men [214]. *KDM6A* is mutated in several cancers, but the highest rates of mutation (20-29%) are found in UBC, with most of the genetic alterations being pathogenic nonsense, frameshift, or splice site mutations [216-219]. In a link between urothelial chromatin organization and the immune system, Kobatake et al [220] found that urothelial-specific *KDM6A* knockout increased expression of several immune genes, most notably genes encoding the *CCL2* and *CXCL1* chemokines, which attract immune cells, and *IL-6*, which induces inflammation [220]. Using a breast cancer cell line model, implanted *KDM6A* knockout cells attracted more non-inflammatory “M2” Mφ, which are believed to foster blood supply and tumor growth [102, 220]. Patient findings correlated with findings in mice. Muscle-invasive UBC with low *KDM6A* expression had greater *CCL2* and *IL-6* RNA and a greater infiltration with M2-type Mφ [220]. An exciting finding is that loss of *KDM6A* in a UBC xenograft model predisposed tumor cells to epigenetic therapy, which diminished in vivo tumor growth and increased natural killer cell attack [219]. In addition to *KDM6A*, Dunford et al [221] found 5 other X-linked genes (*ATRX*, *CNKSR2*, *DDX3X*, *KDM5C*, and *MAGEC3*) with loss of function mutations or copy number changes that were more frequent in men than in women, in many cancer types. This sex-linked association was highly significant, as it was found in 6 out of 783 X chromosome genes but in zero of 18,055 genes from autosome chromosomes or pseudoautosomal X chromosome regions [221]. Although we do not fully understand how most of these X chromosome genes contribute to cancer, their incomplete allelic inactivation may protect women. It is likely that unequal X chromosome gene expression contributes to male UBC

incidence, in addition to social differences, sex hormones, and other consequences of biological sex.

The Y chromosome also affects cancer risk, but functions differently [222]. From analysis of 1,153 elderly men, partial loss of Y chromosome in peripheral blood was associated with increased non-hematological cancer mortality. Men who had lost the Y chromosome had a median survival time of 5.5 years shorter than patients who had intact Y chromosome [222]. More relevant to this discussion, the Y chromosome is uniformly missing from many male UBC [223]. In other cancers with a defective *KDM6A* allele on the X chromosome, the Y chromosome may be present in the cell, but may have extreme downregulation of gene expression [218]. These findings suggest the possibility of one or more Y-linked tumor suppressor genes. If this speculation is confirmed, such a tumor suppressor gene might be uniquely encoded on Y or it could be from the pseudoautosomal region that is shared by X and Y chromosomes [197]. One candidate Y chromosome tumor suppressor gene is *KDM6C* (also known as *UTY*), which is a paralog of *KDM6A*. Although *KDM6C* protein has little histone demethylase activity, it functions as a tumor suppressor in vitro [224, 225] and rescues embryonic development in male *KDM6A* knockout mice [225]. Both *KDM6A* and *KDM6C* proteins act as molecular scaffolds to assemble chromatin-modifying complexes, regardless of their own enzymatic activity [226]. Ahn et al [224] found that 23% of male UBC had deleted *KDM6C*; the *KDM6C* deletion rate was 67% in UBC that also had an X chromosome *KDM6A* mutation. Similarly, Ler et al [217] found *KDM6C* copy number loss in 12% of male UBC and this was more common in UBC that carried *KDM6A* mutations. Thus it would appear that the X chromosome *KDM6A* and the Y chromosome *KDM6C* each have tumor suppressor activity and can partially compensate for each other in males, but that *KDM6A* protein has enzymatic activity that allows it to more powerfully suppress cancer development and growth. The X chromosome *KDM5C* and the Y chromosome *KDM5D* gene products also affect chromatin structure and may modify cancer risk, although their role in UBC has not been documented [218].

10. Sex Differences Not Directly Attributed to Hormones or Sex Chromosomes

Some sex-related differences do not appear to be directly related to sex hormone differences or sex chromosomes. For example, chromosome 3 *VGLL3* shows female biased expression in skin, salivary gland, and in monocytes [227]. Keratinocytes from female subjects expressed more *VGLL3* RNA, whether there was estradiol, testosterone, or no sex hormone in

culture [227]. *VGLL3* is of interest as it may act as a tumor suppressor gene in epithelial ovarian cancer [228], but *VGLL3* expression is a marker for poor outcome in stomach adenocarcinoma and in PCa [229, 230]. Therefore, *VGLL3* is differentially expressed by sex in some tissues and alters tumor outcome. In another example, Sabag et al [231] found that hemizygous deletion of the chromosome 10 *EGR2* (Krox20) gene, whether in whole animals or in myeloid cells, increased bone resorption by osteoclasts in female mice, but not in male mice. The phenomenon continued to be manifest in preosteoclast cell lines cultured several days in vitro in the absence of any sex hormones. However, further investigation [231] showed that the sex differences were manifest only in preosteoclast cells that had been derived from post-pubertal mice (8 weeks old) but not harvested from mice before puberty (4 weeks old). These and other data [232, 233] suggest that sex hormones cause epigenetic differences in the genomes of male and female animals, which persisted long after sex hormones are removed. It is relevant to note that most genes which show sex-specific expression are driven by distinct sets of transcription factors in male and female tissues, even when the transcription factor levels do not vary by sex [234]. This strongly suggests that the sex differences established different epigenetic landscapes, which persisted after direct sex hormone action. Many of the differentially expressed proteins are involved in methylation and presumably control epigenetic gene expression [234].

11. UBC and Natural Killer Cells

NK cells are the most common leukocyte in UBC tumors [235]. Using gene expression data to deduce immune cell infiltration, several groups reported that the presence of NK cells predicts better UBC outcomes [219, 236-239]. Importantly, the level of CD56^{bright} NK cells correlated with both cancer-specific survival and overall survival [235]. These data are consistent with the idea that NK cells slow UBC progression. This idea is reinforced by findings that epigenetic therapy of *KDM6A*-deficient UBCs caused NK cell attack and tumor regression in a preclinical model [219]. As mentioned above, superficial UBC is often treated with intravesical BCG instillation [55-57]. Now used for more than 40 years, BCG intravesicular infusion is the longest continuously-used cancer immunotherapy [55-57]. NK cells are among many immune and non-immune cells that are required for BCG immunotherapy success [55, 240-244]. In other settings, BCG vaccination induces an elevated “trained immunity” against multiple pathogens, including elevated in vitro NK cell responses against non-cross-reactive microbes [245]. Surprisingly, intravesicular infusion of cytokine-activated healthy donor human NK cells were

reported to eliminate orthotopic human UBC mouse xenografts [244]. If this study is confirmed and expanded to human patients, urologists might someday treat superficial UBC with healthy donor activated NK cells.

UBC patient NK cells differ from those of healthy subjects. NK cells from UBC patients expressed low levels of L-selectin (CD62L), which allows lymphocyte circulation through lymph nodes, and low levels of stimulatory NKp30, NKp44, and NKp80 natural cytotoxicity receptors, which are crucial for target cell killing and other NK cell activities [145, 244, 246]. IL-2 boosts NK cell responses in cancer patients [247]. Following subcutaneous IL-2 injection, circulating NK cell number increases [248]. Importantly, some melanomas and kidney cancers respond to IL-2 injections [249]. However, IL-2 is a strong activator of regulatory T cells, which dampen immune responses by both T cells and NK cells [184, 247, 250, 251]. IL-15 is an alternative cytokine because it signals NK cells and memory T cells through the same receptor β and γ chains as IL-2, but it renders effector cells refractory to regulatory T cell suppression [252-254]. Using NK cells as a therapeutic agent, healthy donor NK cells were stimulated in vitro and then infused into hematopoietic stem cell transplant patients, with NK cell responses boosted by IL-2 injection. This combined treatment prevented acute myeloid leukemia relapse [255]. UBC patients have not been infused with NK cells, although intravesicular human NK cell transfer eliminated orthotopic human UBC xenografts in mice [244].

Ideally, cancer immune therapy will target CSC, which drive cancer progression and long-term proliferation by maintaining an undifferentiated state and replacing partially differentiated cancer cells [37, 256, 257]. When a small number of human CSC are transferred into immunodeficient mice, they regenerate a complete tumor, including CSC and more differentiated cancer cells [258-260]. CSC resist radiation and many chemotherapeutic drugs, and account for metastases and post-treatment tumor relapse [37, 256, 257]. Just as Superman was uniquely susceptible to kryptonite, cancers might be uniquely susceptible to NK cells, because they preferentially target CSC [244, 261-264]. Muscle invasive bladder cancers possess a distinct population of CSC, which may contribute to high UBC recurrence rate [265-268]. In various tumors, one or more markers have been used to identify CSC, including CD24, CD44, CD133, Sox2, and aldehyde dehydrogenase [256, 264]. CD24 is significant, because UBC overexpress it compared to normal urothelium [269]. As described earlier, NK cells can be inhibited by MHCI and CSC typically are susceptible to NK cell attack [143, 145, 151, 244, 261-

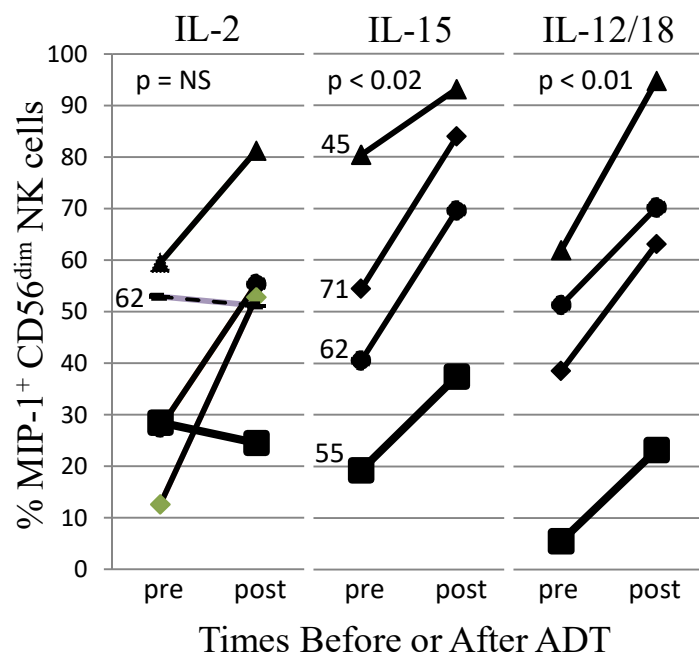
264]. UBC-derived NK cells killed the RT4 UBC cell line much better after blocking MHCI [235], consistent with functional MHCI-specific inhibitory receptors on UBC tumor-infiltrating NK cells. Although CSC may have relatively low or high MHCI expression, CSC are NK-susceptible because they express abundant stimulatory ligands [261-264]. The NK-stimulatory ligands expressed by CSC include NKG2D ligands: MHC class I chain-related protein A and B (MICA and MICB) and ULBP1. NK cell killing of CSC is primarily controlled through the interaction of the NKG2D receptor and its ligands [244, 264]. UBC tumor-derived NK cells also required NKG2D to kill K562 tumor cells [235]. In mouse models, NK cells significantly decreased UBC CSC number [244]. These data suggest that NK cells kill CSC and thereby prevent bladder cancer recurrence.

12. Conclusions

In most non-reproductive tissues, men are more likely than women to develop cancer and die of disease, and UBC is an outstanding example. Gender disparity has many potential causes including androgen-induced immunosuppression, androgen-induced urothelial and neoplastic proliferation, greater male exposure to carcinogens, and the protective effects of X chromosome-linked genes in females. We need to investigate X-linked and Y-linked cancer protective genes, as these might help explain male predominance in most cancers, including UBC. Recognition that UBC is an endocrine tumor could have profound effects on UBC treatment. As previously suggested [44], we might be able to treat recurrent UBC with ADT, similar to PCa. NK cells represent a promising target for immunotherapy in UBC and other cancers. Further research on the role of androgens in NK cell development could help explain cancer gender differences. NK development has been well characterized from hematopoietic stem cell to mature NK cell, but there has not been adequate research on which NK cell developmental stages express AR and are androgen regulated. It is likely that the UBC male predominance is a consequence of multiple factors.

Figure 1. Blood from five patients was studied before and 92-96 days (or 51 days in one patient shown as a dashed line in IL-2 panel) after the start of ADT, which included Lupron[®], with or without additional ADT drugs. In all cases, short-term Casodex[®] pre-treatment was used to prevent a Lupron-associated testosterone flare. Peripheral blood mononuclear cells were stimulated for 20 hours with IL-2 (200 U/mL), IL-15 (100 ng/mL), or IL-12/IL-18 (10/100 ng/mL), as indicated above each panel. The cells were harvested and NK cell intracellular MIP-

1 β was measured by flow cytometry, using standard methods [156]. Shown are responses by mature CD56^{dim} NK cells. Each symbol represents responses by the same patient and patient age is indicated in the panels. Significance was assessed by paired student's t-test. NS = not significant.



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