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Autoimmunity in Coxsackievirus B3 induced myocarditis: role of estrogen in suppressing autoimmunity

SUMMARY

Picornaviruses are small, non-enveloped, single stranded, positive sense RNA viruses which cause multiple diseases including myocarditis/dilated cardiomyopathy, type 1 diabetes, encephalitis, myositis, orchitis and hepatitis. Although picornaviruses directly kill cells, tissue injury primarily results from autoimmunity to self antigens. Viruses induce autoimmunity by: aborting deletion of self-reactive T cells during T cell ontogeny; reversing anergy of peripheral autoimmune T cells; eliminating T regulatory cells; stimulating self-reactive T cells through antigenic mimicry or cryptic epitopes; and acting as an adjuvant for self molecules released during virus infection. Most autoimmune diseases (SLE, rheumatoid arthritis, Grave's disease) predominate in females, but diseases associated with picornavirus infections predominate in males. T regulatory cells are activated in infected females because of the combined effects of estrogen and innate immunity.

Keywords

Autoimmunity; picornavirus; T regulatory cells; estrogen; sex differences

INFECTION AND AUTOIMMUNITY

Microbial infections are strongly implicated as risk factors for the induction of autoimmunity [1], including such diseases as type 1 diabetes [2], rheumatic heart disease [3], myocarditis [4], multiple sclerosis [5], and rheumatoid arthritis/systemic lupus erythematosus [6], but the precise host and microbial characteristics or conditions required for autoimmunity initiation are poorly understood. Three factors are necessary for the development of an autoimmune disease. These are: 1) the presence of a repertoire of autoreactive lymphocytes; 2) activation of the autoreactive cells; and 3) absence of the normal immunoregulatory mechanisms which suppress autoimmunity [7]. T cells are pivotal to the immune response as these cells directly mediate inflammation through release of chemokines and cytokines, and induce target cell cytotoxicity; and provide B cell help for IgG, IgA, and IgE responses. There are several mechanisms by which infections can lead to autoimmunity.

1. Loss of tolerance

The first mechanism is loss of tolerance. Tolerance is induced either centrally in the thymus during T cell ontogeny or peripherally through anergy or induction of regulatory cells. Self-reactive prothymocytes enter the thymus where high affinity interactions between the T cell receptors on the developing T cells and major histocompatibility complex (MHC) antigen on thymic epithelium initiates clonal deletion of autoreactive T cells through apoptosis. Infections with viruses (HIV, rabies), bacteria (*F. tularensis*), parasites (*T. cruzi*, *P. chaubii*, *S. mansoni*, *T. spiralis*) and fungi (*H. capsulatum*, *P. brasiliensis*) cause thymic atrophy by increasing glucocorticoid hormone levels which activate caspases 3 and 8[8,9]. Similarly, infections with coxsackievirus B3, but not with coxsackievirus B1 or other picornaviruses, cause both thymic and peripheral T cell atrophy [10], but in this case glucocorticoid hormones may not be involved as adrenalectomy failed to prevent thymic involution. In addition to apoptosis of T cells, infection alters intrathymic T cell migration resulting in increased release of immature T cell and self-reactive T cells into the periphery [8]. After leaving the thymus, autoreactive T cells

should be subjected to peripheral tolerance. Peripheral tolerance can result either from deletion or anergy of self reactive cells by inappropriate antigen presentation [11]. Anergy is a functional state in which T cell proliferation and cytokine production are suppressed, and is usually attributed to inhibition of signal transduction subsequent to T cell receptor (TCR) and co-stimulatory molecule (CD28) engagement [12]. Anergized T cells show impaired tyrosine kinase phosphorylation of the T cell receptor and blocked phosphorylation of signal kinase (MAPK, ERK, JNK) pathways downstream of the T cell receptor [13,14]. Anergized cells remain alive for extended periods, but are incapable of responding even when the appropriate antigen is presented by professional antigen presenting cells. Recently, other mechanisms of T cell anergy have been proposed. Studies indicate that the cell cycle inhibitor p27^{kip1} is increased during anergy and that over-expression of this molecule induces an anergic state [15,16], although whether p27^{kip1} induces anergy through prevention of T cell proliferation or through promoting Smad3 dependent transcription is controversial [17]. In addition to activation of downstream kinases, CD28 increases cellular metabolism including glucose and amino acid uptake and protein synthesis through Akt and mTOR (mammalian target of rapamycin) [18]. Blocking cellular metabolism anergizes T cells despite normal engagement of TCR/CD28 and signal transduction [18]. Finally, anergy can be actively induced in T cells through PD-1 (programmed death-1)/PD-L1 interactions. PD-1 is an inhibitory molecule belonging to the CD28 family, which is up-regulated on T cells after antigen stimulation and inhibits T cell proliferation and cytokine production [19]. Furthermore, PD-1/PD-L1 interactions induce T cell anergy through suppression of IL-2 response [20]. PD-1/PD-L1 interactions and T cell anergy are important in myocarditis [21–23]. Mice deficient in PD-1 spontaneously develop myocarditis [22] and treating coxsackievirus B3 infected animals with agonist anti-PD-1 antibody augments both myocardial inflammation and pro-inflammatory cytokine expression [23].

It is unlikely that viral infections rescue anergized autoimmune T cells through inhibition of MAPK/ERK since many virus infections, including picornaviruses, are known to activate these pathways [24,25]. p38 MAPK activation is necessary for maximal virus production, and most likely is essential for progeny virus release since viral protein synthesis is not affected by inhibition of p38MAPK. The mechanism for virus-induced MAPK/ERK activation during picornavirus infection probably depends upon functional lipid rafts which are required for virus internalization and targeting of the virus to the golgi [26], but are also implicated in MAPK/ERK activation [27]. If viruses are unlikely to rescue anergized autoimmune T cells through suppression of p38MAPK, they might be highly effective in promoting autoimmunity through production of IL-2. High levels of IL-2 reverse T cell anergy and can potentially lead to autoimmunity [28]. Infection of mice with non-myocarditic variants of coxsackievirus B3 induce little IL-2 while infections with myocarditic virus variants stimulate strong IL-2 responses [29]. Injection of exogenous recombinant IL-2 into mice infected with non-pathogenic coxsackievirus B3 restores myocarditis susceptibility suggesting that viral myocarditis requires IL-2 to reverse T cell anergy and that only virus variants capable of promoting good IL-2 responses will induce this disease.

2. Loss of T regulatory cells

Diverse populations of lymphoid cells have the capacity to inhibit immune responses. Although most reports center on CD4⁺ T regulatory cells, CD8⁺ T regulatory cells are similar both in their generation and function [30,31]. With each cell population (CD4⁺ or CD8⁺), immunosuppressive cells can be divided into natural and inducible populations.

Natural T regulatory cells arise from the CD4⁺CD8⁺ developmental stage by induction of both CD25 and Forkhead box P3 (FoxP3) transcription factor; the latter factor sustaining and stabilizing the regulatory activity of these cells [32]. FoxP3 binds to approximately 700 genes

[33]. Among its other functions, FoxP3 blocks the ability of NFAT and NFkB to activate their target genes resulting in transcriptional suppression of cytokine (IL-2, IL-4, IFN γ) expression [34–36]. Generation of the natural T regulatory cells involves CD28, CD40L, and LFA-1 expression on the precursor T cells and CD80, CD86, CD40 and ICAM-1 expression on thymic stroma [37] while IL-2, IL-7 and IL-15 maintain T regulatory cells in the periphery [32,38]. In contrast to the natural T regulatory cells, inducible T regulatory cells arise in the periphery through antigen activation of naïve CD4⁺ cells [39]. Naïve CD4⁺ cells stimulated in the presence of TGF β differentiate into inducible T regulatory cells while naïve CD4⁺ cells stimulated in the presence of IL-6 and possibly IL-23 differentiate into Th17 cells [39,40]. IL-23 may not be required for the differentiation of Th17 cells but may be necessary for Th17 cell survival [41]. Th17 cells express retinoic acid orphan receptor- γ t transcription factor, IL-17A, IL-17F and IL-22 and increase expression of IL-1, IL-6 and G-CSF and enhance autoimmunity in both humans and animals [40]. Diseases associated with Th17 include rheumatoid arthritis and multiple sclerosis [42]. As with natural T regulatory cells, many inducible T regulatory cells express FoxP3 [31].

Natural T regulatory cells primarily suppress immunity through direct cell-cell contact using CTLA-4 or membrane bound TGF β [32,43]. Inducible T regulatory cells can suppress immunity by CTLA-4 as in natural T regulatory cells or through production of IL-10 or TGF β . T regulatory cells can also inhibit effector T cell responses through metabolic disruption or killing of T cells, alteration of antigen presenting cell function, induction of indoleamine 2,3-dioxygenase (IDO), and physical impedance of effector T cell interaction with antigen presenting cells [31].

T regulatory cells have a crucial role in determining myocarditis susceptibility to coxsackievirus B3 infection [44,45]. Reducing CD80 expression on mast cells and macrophage in virus infected mice results in decreases in CTLA-4 expression and T regulatory cell numbers with subsequent increases in cardiac inflammation [44]. Furthermore, the relative ability of coxsackievirus variants to induce T regulatory cells may be pivotal in whether the infections are pathogenic or non-pathogenic. Studies from this laboratory using two coxsackievirus B3 variants which differ by a single nonconserved amino acid in the VP2 capsid protein [46] show that the non-pathogenic variant induces a potent T regulatory cell (CD4⁺CD25⁺FoxP3⁺) response which is absent during infections with the pathogenic virus [45]. Similar results have been found in coxsackievirus B4 infection where induction of T regulatory cells prevents insulin dependent diabetes [47]. Cytokine responses during infection, involving either IL-13 [48] or TGF β [47], promote T regulatory cell generation.

In infectious diseases, induction of T regulatory cells might be detrimental as suppression of anti-pathogen immunity could increase microbial replication and direct pathogen induced tissue injury. In this case, induction of T regulatory cells could lead to chronic infections while lack of a T regulatory cell response would allow rapid control and elimination of the infectious agent [49,50]. However, in coxsackievirus infection, induction of T regulatory cells is not associated with chronic infection [45] [47]. Virus elimination occurs normally in animals having enhanced T regulatory cell responses even though immunopathogenic injury is ablated. In part, suppression of T cell immunity has minimal effect on acute picornavirus infections [51] because T-cell independent responses (including neutralizing antibody [52], RIG-1/MDA-5 helicases and type 1 interferons [53], and nitric oxide [54]) are highly effective in clearing the virus.

3. Antigenic mimicry

Another mechanism for infection induced autoimmunity is antigenic mimicry in which microbial and self-molecules share sufficient similarity to induce cross-reactive immunity [55–57]. Cross-reactive T cells or antibodies between microbes and self antigens are found in

various autoimmune diseases including: ankylosing spondylitis, between HLA B27 and *Krebsiella pneumonia* [58,59]; myasthenia gravis, between the acetylcholine receptor and herpes simplex virus [60]; type I diabetes, between glutamic acid decarboxylase and human cytomegalovirus [61]; rheumatic heart disease, between the M protein of group A streptococcus and cardiac myosin [62]; multiple sclerosis, between myelin basic protein and Epstein Barr Virus DNA polymerase [63]; and Sjogren's syndrome, between the Ro60 kD autoantigen in salivary gland and hepatitis C virus [64]. Antigenic mimicry has been implicated between picornaviruses and self molecules in Sjogren's syndrome [58,59,65–67] involving cross-reactivity between coxsackievirus and the Ro/SSA 60 kD autoantigen; in type 1 diabetes [68] between coxsackievirus and the glutamic acid decarboxylase (GAD65) autoantigen [64,69, 70]; in myocarditis [71] between coxsackievirus and cardiac membrane proteins.

When cross-reactive antibodies but not T cells are observed [66,72], mimicry probably involves the tertiary conformation of the antigens. In contrast, cross-reactive T cell clones [61] usually recognize similar primary amino acid sequences between microbes and self antigens since T cells respond to peptides presented by MHC molecules. Although one often is told that a single T cell clone responds to a specific epitope of 8-20 amino acids presented by a major histocompatibility complex (MHC) molecule [73], in fact, there is significant flexibility in the T cell receptor recognition of the epitope. Subtly altered peptides may partially or fully initiate T cell activation which may lead to cytokine production without T cell proliferation or to anergy [74–76]. Distinct structural characteristics of the epitope determine its ability to functionally interact with the TCR and stimulate immunity [77] so that of the 8-20 amino acids in the total peptide, only amino acids at specific positions will be determinative and substitution of other amino acids at these residues which are equivalent in size and/or polarity can stimulate the same T cell response. Thus, very limited sequence homology between the microbial and self antigens may be required for cross-reactivity [63,74] increasing the probability for antigenic mimicry.

4. Cryptic epitopes

Cryptic epitopes are immunogenic peptides which are either not generated during normal antigen processing or generated at too low concentrations for effective T cell activation [78]. Low cryptic epitope generation during normal antigen processing may result from cleaving of proteins within potential cryptic epitopes by the proteases normally present in antigen presenting cells. Concentrations of cryptic epitopes might be augmented by increasing the amount of the self protein degraded. Although most cryptic epitopes will still be destroyed, sufficient amounts may escape to allow immune sensitization [78]. Virus-induced ubiquitination of self proteins in infected cells can lead to increased self molecule degradation such as occurs with coxsackieviruses that downregulate cellular cyclin D1 using the ubiquitin-proteasome pathway [79]. Low levels of cryptic epitopes may also be made more antigenic by increasing efficiency of their presentation to T cells through up-regulation of major histocompatibility complex (MHC) and accessory molecules on antigen presenting cells or by release of pro-inflammatory cytokines required for T cell proliferation [78]. The types of proteases can also vary among distinct antigen presenting cells which might produce distinct peptide repertoires for T cell activation [80]. IFN γ and TNF α exposure alter protease expression in cells and may change the peptides produced [81]. Protein-protein interactions can affect antigen processing. For example, internalization of HIV-CD4 complexes results in cryptic epitope production of the CD4 molecule and autoreactivity [82,83]. Similarly, antigen-antibody complexes might alter protease access to cleavage sites in antigens during antigen processing [84]. FcR up-take of immune complexes could deliver antigen to different compartments of the endosome pathway which may also alter antigen processing [85]. Finally, virus infections may cause cryptic epitopes generation in self proteins through the action of viral proteases. Many viruses, as well as other infectious agents, code for their own proteases

which are necessary for processing of microbial proteins. For example, the picornavirus genome consists of a single strand RNA which contains 5' and 3' non-translated regions and a single open reading frame. The open reading frame is translated into a single polypeptide which is processed by viral proteases into at least 11 proteins. Picornavirus proteases not only cleave the viral polypeptide, but also degrade the host eukaryotic initiation factor 4G (eIF-4G) which disrupts the cellular p220 protein cap-dependent binding complex and results in the shutdown of cap-dependent host protein synthesis [86]. This is an important adaptation by the virus which allows it to shut down host cell protein synthesis for maximum progeny virus production. Viral proteases additionally cleave cellular proteins not required for virus replication such as dystrophin, a contractile protein in myocytes [87]. Cleavage of dystrophin itself could result in cardiomyopathy [87], but it is also likely that the viral protease cleaves dystrophin differently than cellular proteases and this could result in cryptic epitope generation.

5. Adjuvants and autoimmunity

Adjuvants are compounds which, when concurrently administered with antigen, substantially enhance the immune response to that antigen [88,89]. The crucial aspect of a good adjuvant is to rapidly activate the innate immune system to prime antigen presenting cells for antigen uptake, maturation and optimal antigen presentation to the adaptive immune system along with elicitation of pro-inflammatory cytokines [90]. Often, this employs use of microbial components in the adjuvant, such as Mycobacterium, which activate the innate immune system through toll-like receptors (TLR). TLR are type I integral membrane glycoproteins which have extracellular domains with variable numbers of leucine-rich repeat (LRR) motifs and conserved intracellular domains with a homologous Toll/interleukin (IL)-1 response domain [91,92]. There are 12 TLR in humans and mice which react to different molecular species [41,91,93–96]. TLR are pattern recognition receptors (PRR), cellular molecules which recognize pathogen-associated molecular patterns (PAMPs). PAMPs are conserved microbial molecules shared by a diverse population of pathogens and are probably required for microbial infection or replication [91]. The ability of an individual to recognize PAMPs allows a very rapid host response to infection without requiring specificity of the response to a particular infectious agent. TLRs can be expressed either individually or as heterodimers (TLR1/TLR6, TLR1/TLR2 or TLR2/TLR6) [91–93]; and different cells express distinct sets of TLR. For example, plasmacytoid dendritic cells express TLR7 and TLR9 but not TLR3; monocyte-derived dendritic cells express TLR3 but not TLRs7 and 9; CD8+ cells express high levels of TLR3 but very low levels of TLR2 and TLR4; and B cells express high levels of TLR9 and TLR10 [93,97,98]. However, TLR expression and functionality is dynamic and can change with the activation state of the cell. TLR2 and TLR4 mRNA and intracellular protein are expressed by naïve CD4+ cells but these TLR proteins are not present on the plasma membrane. After activation, CD4+ cells rapidly express cell-surface TLR2 and TLR4, but only the TLR2 may be functional resulting in IFN γ , IL-2 and TNF α expression [99]. Evidence indicates that both the type and intensity of signal transduction through specific TLRs determine the nature of the adaptive immune response that they promote. TLRs 3, 4, 5 and 9 preferentially activate Th1 responses while TLR2 preferentially induces Th2 immunity [98]. Similarly, while low dose LPS stimulation of TLR4 promotes Th2 response, higher doses of LPS promote Th1 responses. This means that distinct lymphoid cell populations will respond differently in infections depending upon both the TLR ligand(s) of the invading organism and the TLR subsets expressed by the responding lymphoid cells.

Many experimental diseases can be induced either through injection of mice with TLR activating molecules such as double stranded RNA or lipopolysaccharide alone (hepatitis) [100]; or, more often through injection of tissue homogenates/proteins in adjuvant (experimental allergic encephalitis; thyroiditis, myocarditis) [101–103]. This strongly implicates an adjuvant role in induction of various tissue specific autoimmune diseases, at least

experimentally, and provides strong circumstantial evidence that a major role for viruses and other microbes in autoimmunity induction is their ability to stimulate strong TLR signals. Infections are often implicated in the induction of autoimmune diseases but the mechanism(s) by which infection results in autoimmunity are controversial [57,104]. Although the concept of antigenic mimicry has been around for decade, the definitive evidence that this plays a decisive role in autoimmune diseases is still lacking. However, recent studies in coxsackievirus B3 induced myocarditis have shown that TLR4 deficient mice develop significantly less myocarditis demonstrating a pivotal role for TLR signaling in this viral induced disease ([105]; reviewed by Rose [104]). One question might be why TLR4 should be important in coxsackievirus B3 induced myocarditis when this virus is non-enveloped and would therefore lack the classical TLR4 ligand, lipopolysaccharide. However, it is now clear that coxsackievirus proteins can directly bind and activate TLR4 [106]. Also, it is now known that endogenous as well as microbial ligands can activate TLR and that the endogenous ligands may act as “danger” signals to the innate immune system [107–110]. Cardiac myosin has recently been shown to activate TLR2 and to cause cardiac inflammation [110–112]. Furthermore, coxsackievirus infection increases TLR2, 7 and 8 mRNA expression [113]. Since coxsackievirus B3 is lysogenic, it is reasonable to assume that virus-induced death of cardiac myocytes results in release of cardiac myosin peptides and potential activation of TLR2 through this endogenous ligand.

TLR signal transduction can either positively or negatively affect T regulatory cell activity. TLR8-MyD88 signaling suppresses T regulatory cell function, while TLR2 and TLR5 both increase T regulatory cell numbers and activity [41,114,115]. How TLR signaling modulates T regulatory cells is complex, but most likely results via alterations in dendritic cell cytokine or accessory molecule expression [116,117]. Activation of NFkB and production of pro-inflammatory cytokines (IL-1 β and TNF α) subsequent to TLR signaling may be crucial in inducing autoimmunity through the effects of these cytokines on antigen presenting cells or directly on T regulatory cells [118,119]. IL-1 β and TNF α promote dendritic cell maturation and migration and also up-regulate expression of CD80 and CD86 [118]. While these effects should enhance immunogenicity of self antigens and promote autoimmunity, they may also alter T regulatory cells. Mice lacking CD80, CD86 and CD40 have fewer T regulatory cells than wild-type mice [115]. Therefore, one might expect that IL-1 β and TNF α should enhance T regulatory cell responses because these cytokines increase CD80/CD86 expression. However, TNF α directly interacts with T regulatory cells independent of antigen presenting cells and suppresses their function in hepatitis B virus infections [119]. In contrast to TNF α , IL-2, IL-4 and IL-7, which are also generated from TLR activation, strengthen the T regulatory response [34,117]. In addition to effects mediated through cytokines, direct TLR signaling in T regulatory cells could modulate immunosuppression [114]. Human T regulatory cells express TLR4, 5 and 8 and do not express TLR7. Other TLR have not been determined. Mouse T regulatory cells express TLR1, 2, 4, 5, 6, 7, and 8 but not TLR3 or 9 [114,120].

SEX AND PICORNAVIRUS-INDUCED AUTOIMMUNITY

Females are often less susceptible to viral infections than males. This is true for a wide range of viruses including picornaviruses (coxsackievirus, Theiler’s virus, encephalomyocarditis virus), Herpes Simplex virus type 1, HIV, hantaviruses, VSV, and West Nile Virus (see [121]). The reasons why females are less susceptible are complex but most likely relate to the enhanced immune responses occurring in this sex. Estrogen modulates both innate and adaptive immunity. Although some effects of estrogen on immunity are controversial, the following are generally accepted: increasing immunoglobulin synthesis[122]; suppressing both T and B cell lymphopoiesis [123]; enhancing dendritic cell differentiation and antigen presentation[124]; suppressing TNF α and IL-6 expression [125,126] while increasing IL-4 and IFN γ production [127,128]; inhibiting B cell apoptosis[129]; and promoting FoxP3+ T regulatory cell

development [130–132]. The effects of estrogen on immune responses and cytokine expression can be controversial. Although the majority of studies indicate that estrogen increases IFN γ expression, there are several reports that estrogen either has no effect on IFN γ [127] or decreases its expression [133]. In coxsackievirus B3 infections, estrogen suppresses IFN γ and Th1 responses while promoting IL-4 and Th2 responses [134]. The reason for the divergent effect of estrogen on IFN γ induction during coxsackievirus B3 infection relates to the suppressive effects of this hormone on the virus receptor (discussed below) which restricts virus infection and replication and therefore indirectly affects cytokine and immune responses (see Figure 1). In this case, the effect of estrogen on the immune response becomes secondary to its effects on virus infection.

Part of the complexity associated with estrogen in viral infections is that this hormone alters both nuclear and non-nuclear signal pathways. There are two known estrogen receptors (ESR1 and ESR2) which bind with equivalent specificity and affinity to estrogen response elements (ERE) in gene promoter regions leading to modulation of gene expression [135]. ESR1 (also known as ER α) and ESR2 (also known as ER β) have wide tissue distribution with ESR1 primarily found in the uterus, liver, kidney and heart while ESR2 is primarily found in ovary prostate, lung, central nervous system, gastrointestinal tract, and bladder. Lymphocytes, macrophage and dendritic cells co-express both ESR1 and ESR2 [136] but ESR1 primarily controls estrogen modulation of dendritic cell maturation, T cell cytokine production and immunoglobulin response [126,127,137,138]. In contrast, signaling through ESR2 up-regulates inducible nitric oxide synthetase (iNOS) and nitric oxide generation while ESR1 suppresses this response [139]. Several important studies now show that where ESR1 and ESR2 are co-expressed in the same cell, these receptors may exert opposing effects on gene expression and thus counter-balance each other [133,135]. Since there is clearly differential tissue distribution of the nuclear receptors, it is quite possible for estrogen to have divergent effects depending upon where a virus infection occurs. For example, since ESR1 is more prevalent in heart while ESR2 is more prevalent in lung [135], one might expect different estrogen effects in cardiotropic coxsackievirus infection than in influenza virus infections which are restricted to the respiratory tract. Although the genomic effects of estrogen receptors have been most extensively studied, it is now clear that ESR1 can also translocate from the nucleus to the plasma membrane resulting in modulation of intracellular calcium flux and activation of both the MAPK/ERK and PI3K/AKT pathways. Both ESR1 and ESR2 can also bind to mitochondria DNA and modulate mitochondrial respiratory complexes and production of reactive oxygen species [140].

For some viruses and specifically for coxsackieviruses, estrogens can directly affect virus infection and replication independent of its effects on immunity. Viruses use cell surface molecules for attachment to cells and initiation of the infection cycle. At least some molecules used as virus receptors are under estrogen control. These include CXCR4 which can act as a co-receptor for HIV; $\alpha v \beta 3$ integrin which acts as a receptor/co-receptor for adenovirus, coxsackievirus A9 and hantavirus [141–145]; and decay accelerating factor (DAF; CD55) which is one of the two known receptors for coxsackieviruses. The other known receptor is coxsackievirus-adenovirus receptor (CAR) [146–148]. Although most coxsackieviruses use both CAR and DAF for optimal infection [149], CAR appears to be the dominant receptor and can lead to infection of cells in tissue culture with little or no DAF involvement [150]. In vivo, receptor usage is more complex and may depend upon the type of cell or tissue infected. Chimeric fusion proteins (DAF-Fc and CAR-Fc) are highly effective in inhibiting coxsackievirus infection in the heart but CAR-Fc alone prevents virus infection of the pancreas indicating that both receptors are required for optimal cardiac infection whereas DAF is not important for infection of the pancreas in the same infected animal [147,151,152]. Studies using DAF knockout mice confirm that this receptor is important for myocarditis but not pancreatitis [153]. DAF expression is enhanced by estrogen treatment in a tissue specific

manner [154], but only recently has it been shown that ESR1 and ESR2 have opposite effects on DAF expression. ESR1 suppresses and ESR2 increases DAF on lymphoid cells. Increased DAF also correlates to enhanced susceptibility to develop myocarditis (Huber, manuscript in preparation) [155]. The effect of estrogen on DAF expression seems at odds with the resistance of females to coxsackievirus B3 induced myocarditis [155]. The primary function of DAF is to inactivate the C3 component of complement and protect cells from complement mediated lysis [156]. However, DAF has been shown to suppress T cell responses during allograft rejection [157] and depletion of DAF can promote autoimmunity [158]. The mechanism by which DAF suppresses T cell immunity is not well understood although it apparently requires complement, the natural ligand of DAF. It is not clear whether estrogen also increases CAR expression, although this is quite possible since estrogen inhibits TNF α responses and TNF α and IFN γ both suppress CAR expression [159]. What direct effects modulation of CAR expression might have on innate or adaptive immunity is not clear. However, CAR is a junction adhesion molecule (JAM), proteins which are located in tight junctions and are essential in structural stability of the junction [160,161]. In addition, JAMs are involved in leukocyte transmigration through endothelial barriers. When endothelial cells are exposed to cytokines, JAMs re-distribute outside of tight junctions and bind to specific leukocytes enabling their transmigration [160]. Since CAR binds immunoglobulin [162], it is probable that this tight junction molecule promotes B cell transmigration. Increasing CAR expression could reinforce tight junction stability and inhibit B cell transmigration into underlying tissues; both effects would result in reduced inflammation.

Substantial evidence indicates that tissue injury in picornavirus infections results from immunopathogenic mechanisms rather than from direct virus induced killing of infected cells. T cell depletion of mice prevents myocarditis, diabetes and demyelination in coxsackievirus B3 [51], encephalomyocarditis virus [163], and Theiler's virus [164,165] infections. In coxsackievirus B3 infections, cytolytic CD8 $^{+}$ T cells reactive to myocyte antigens are the primary mediators of cardiac injury [166–168]. With coxsackievirus induced myocarditis, the basis of the autoimmune response appears to be antigenic mimicry between the virus and cardiac proteins including cardiac myosin [3,71,169]. However, the existence of mimicking epitopes is not sufficient for induction of autoimmunity. Variants of coxsackievirus B3 which contain functional mimicking epitopes may not generate autoimmunity unless T regulatory cell responses are prohibited. As indicated above, coxsackievirus B3 infection of female mice fails to generate autoimmunity while male mice are highly susceptible [170,171]. Sex associated hormones control cardiac pathology since castration of males is protective while restoration of testosterone increases susceptibility [29,170,172,173]. Furthermore, treating male mice with 17- β -estradiol significantly suppresses myocarditis while treatment of females with androgen enhances myocarditis [171]. Estrogen protects against coxsackievirus B3 induced myocarditis and autoimmunity by multiple mechanisms. As discussed above, reduction in virus receptor expression would limit virus load and consequently activation of innate and adaptive immunity. A second mechanism for protection results from the ability of estrogen to promote CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$ T regulatory cell response [45,174]. In the CVB3 induced myocarditis model, signaling through the ESR1 is responsible for T regulatory cell response while signaling through ESR2 inhibits T regulatory cell activation (Huber manuscript submitted). This observation is consistent with previous published reports showing the same phenomenon [132,175].

The mechanism(s) for enhanced T regulatory response in females could be quite complex. One possibility might be an inherent increase in FoxP3 expression in females. Mice either lacking FoxP3 or having mutated forms of this transcription factor are highly susceptible to autoimmunity [176]. Although FoxP3 is located on the X chromosome [33], it is unknown whether females inherently express more of this transcription factor than males because of X chromosome inactivation. In placental mammals, the X inactivation center located on the X

chromosome contains an Xist RNA gene which triggers chromosome wide gene repression and maintains gene dose balance between males and females [177]. Some genes escape X inactivation [178]. The number of X chromosome genes which escape is not clear but may be as few as 5% of the genes on this chromosome [179]. Whether FoxP3 is one of the few X-linked genes which escapes inactivation and may therefore be expressed at higher levels in females is not known. More likely epigenetic control of FoxP3 expression is more important in T regulatory cell responses. The FoxP3 gene contains 11 coding and 3 non-coding exons, and intronic enhancer, proximal promoter and upstream enhancer region [180]. Various extracellular and intracellular signals modulate FoxP3 expression with TGF β , IL-2 and IFN γ promoting expression and interferon regulatory factor 1 (IRF-1), IL-6 and IL-4 suppressing expression. In many cases control of FoxP3 transcription depends upon CpG methylation of specific sites in the promoter and intronic enhancer regions. For example, IL-6 induces methylation of FoxP3 through STAT3 dependent mechanisms resulting in suppressed FoxP3 expression. Fully demethylated FoxP3 as found in natural T regulatory cells can result in stable suppressive activity. In contrast, adaptive peripheral T regulatory cells can be more readily re-methylated at the FoxP3 gene leading to loss of suppressive function. Estrogen increases expression of both TGF β and IFN γ [181] while decreasing expression of IL-6 [182]. Thus, estrogen both provides extracellular stimuli which promote FoxP3 transcription and eliminates IL-6 dependent methylation of the FoxP3 gene.

CONCLUSION

Viruses, and other microbial infections, have long been associated with autoimmune diseases. Infections can promote autoimmunity through multiple mechanisms and most likely, different mechanisms will be involved with different viruses. In coxsackievirus B3 infections, autoimmunity is the major pathogenic response causing myocarditis. The basis of the autoimmunity is antigenic mimicry between the virus and cardiac proteins, but this antigenic mimicry alone is insufficient for autoimmunity to develop. Environmental factors must be conducive to autoimmune stimulation. These environmental factors include activation of innate immunity, most likely with a strong involvement of toll-like receptor signal transduction, and inhibition of T regulatory cell activation. Although natural T regulatory cells are constitutively present, a potent pro-inflammatory environment could readily negate their immunosuppressive effect and allow autoimmunity induction. Similarly, conditions which strongly favor T regulatory responses might prevent autoimmunity in the face of innate activation. A major difference between males and females infected with coxsackievirus B3 is that the females generate T regulatory cells subsequent to infection which the males lack. The failure of males to develop T regulatory cells probably results first from the potent TNF α and IL-6 responses induced in this sex, both cytokines suppress T regulatory cells. Secondly, increased TGF β expression in females would promote T regulatory cell development. A second method in which females could suppress immunopathogenic responses is through estrogen induced increases in DAF expression. The role of DAF in coxsackievirus B3 infections is likely to be highly complex as DAF is not only capable of modulating T cell responses but is also one of the known receptors of coxsackievirus B3. Total lack of DAF expression in the heart prevents optimal virus infection of this organ. However, signaling through DAF on T lymphocytes is immunosuppressive. This means that low level expression of DAF should promote coxsackievirus B3 induced myocarditis by allowing virus infection of the cardiac myocytes but that substantial increases in DAF expression caused by estrogen should be immunosuppressive and prevent immunopathogenic, T cell-dependent cardiac injury.

TLRs undoubtedly play a central role in coxsackievirus B3 pathogenicity. Their role is most likely multifactorial as they would promote activation of the autoimmune T cells and simultaneously suppress T regulatory cell responses through production of TNF α and IL-6. Although this investigator has only identified autoimmune T cells which react to mimicking

epitopes between coxsackievirus B3 and cardiac antigens, signaling through TLR should also promote autoimmunity to self antigens released during virus induced death of infected cardiocytes in what has been termed an “adjuvant effect”. Similarly, it is very reasonable to assume that cryptic epitope induced autoimmunity could be important in coxsackievirus immunopathology. Although multiple mechanisms for the generation of cryptic epitopes have been postulated, it seems that a highly probable mechanism, production of unique sets of peptides through microbial protease cleavage of self proteins during infection, is rarely, if ever discussed. The ability of coxsackieviral proteases to efficiently cleave self proteins is well known and it seems probable that viral protease cleavage sites could differ from those of endogenous cellular proteases. Considering the substantial amount of virus protein production, significant levels of cryptic peptides should result during viral replication in cardiocytes. The cryptic epitopes, coupled with the adjuvant effect of infection, should be a potent engine for activation of autoimmune T cells. In infections which fail to induce autoimmunity, presumably factors such as induction of immunosuppressive cytokines, modulation of antigen presenting cells, or induction of T regulatory cells prevent the autoimmune response from occurring.

Future Prospective

Virus induced autoimmunity clearly occurs both clinically and in experimental disease models. The mechanisms for autoimmunity induction are highly controversial, however, with different investigators proposing various mechanisms even for the same virus. Thus, for coxsackievirus B3, distinct investigators have forwarded adjuvant effect, cryptic epitope and antigenic mimicry as the primary method for eliciting autoimmune responses. In fact, all of these mechanisms can lead to autoimmune T cell activation, but none of these processes will result in autoimmune *disease*, unless immunosuppression is eliminated. Clinically, understanding how to restore T regulatory cell responses in patients with post-infectious autoimmunity/immunopathology may be more beneficial than specifically defining how activation of autoimmune T cells occurred in the first place. Mechanisms for promoting or eliminating T regulatory cells will differ dramatically depending upon the characteristics of the virus infection and host response to infection. Because different viruses can induce distinct innate responses, it is reasonable to assume that specific conditions favoring immunosuppression in one infection may not exist in another infection. In coxsackievirus B3 infection of mice, sex is a crucial factor promoting T regulatory cell activation but the processes leading to T regulatory cell response are not understood. A major area for future investigation will be to define how why some viruses induce autoimmunity while others do not.

Executive summary

- Infections can stimulate auto-reactive T cell clones by a number of different mechanisms
- More than one mechanism for activating auto-reactive T cells may occur in any specific infection
- Activating auto-reactive T cells is not sufficient to produce autoimmune disease, however. Elimination of immunosuppression (T regulatory cells) is a necessary element in autoimmunity.
- Unlike most autoimmune diseases (SLE, rheumatoid arthritis, Grave’s disease) which predominate in females, autoimmunity subsequent to picornaviruses predominates in males. The reason for this is that estrogen promotes T regulatory cell responses.

- Estrogen most likely suppresses immunoregulation by inhibiting IL-6 and TNF α production, both of which prevent FoxP3 expression.

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Figure 1.

Effects of estrogen on innate and adaptive immune responses. Estrogen signals through two receptors (ESR1 and ESR2). ESR1 suppresses expression of one of the two known coxsackievirus B3 receptors, decay accelerating factor (DAF) which will restrict virus infection and replication. Lower virus loads will result in less direct virus mediated injury by reducing numbers of infected cells and will also reduce Toll-like receptor (TLR) signaling which will restrict pro-inflammatory cytokine expression. Reduced virus loads will also decrease the amount of viral epitopes which mimic self molecules and thus prevent induction of autoimmunity through this mechanism. ESR1 also promotes antibody responses and increased virus neutralizing antibody will further inhibit virus infection. Finally signaling through ESR1 promotes T regulatory cell responses which will further suppress immunopathogenic T cell activation. ESR2 has the opposite effects of ESR1 as signaling through this receptor increases virus receptor expression/virus infection and suppresses T regulatory cell response.