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Donor CD8 T cell activation is critical for greater renal disease severity in female chronic graft-vs-host mice and is associated with increased splenic ICOS^{hi} host CD4 T cells and IL-21 expression¹

Anthony D. Foster^{*}, Mark Haas[†], Irina Puliaeva^{*}, Kateryna Soloviova^{*}, Roman Puliaev^{*}, and Charles S. Via^{*,2}

^{*} Department of Pathology, Uniformed Services University of Health Sciences, Bethesda MD 20814

[†] Department of Pathology & Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048

Abstract

Lupus-like renal disease in DBA/2-into-F1 (DBA→F1) mice is driven by donor CD4 T cells and is more severe in females. Donor CD8 T cells have no known role. As expected, we observed that females receiving unfractionated DBA splenocytes (CD8 intact→F1) exhibited greater clinical and histological severity of renal disease at 13 weeks compared to males. Surprisingly, sex based differences in renal disease severity were lost in CD8 depleted→F1 mice due to an improvement in females and a worsening in males. CD8 intact →F1 female mice exhibited significantly greater donor and host effector (CD44^{hi}, CD62L^{lo}) CD4 T cells and ICOS^{hi} CD4 T follicular helper cells than males. CD8 depleted→F1 female mice exhibited a reduction in the absolute numbers of host, but not donor CD4 Tfh cells and lost the significant increase in host CD4 effector cells vs. males. Greater female IL-21 expression, a product of Tfh cells, was seen in CD8 intact→F1 and although reduced was still greater than male CD8 depleted →F1 mice. Thus, donor CD8 T cells have a critical role in mediating sex based differences in lupus renal disease severity possibly through greater host ICOS^{hi} CD4T cell involvement.

Introduction

A hallmark of systemic lupus erythematosus is pathogenic autoantibody formation directed against nuclear antigens, particularly chromatin¹. Although B cells are intrinsically abnormal in some murine models of lupus², pathogenic IgG autoantibodies in SLE typically exhibit features characteristic of a normal antigen driven response e.g., somatic mutation, affinity maturation and T cell help^{3–7}. CD4⁺ T cells are central in driving B cell autoantibody production in both human and murine lupus^{8–10}. Although CD8 T cells have been described

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Address correspondence to: Charles S. Via M.D., Department of Pathology, Room 3B100, 4301 Jones Bridge Road, Uniformed Services University of Health Sciences, Bethesda, MD 20814. Phone: 301 295 3801; Fax 301 295 1640, cvia@usuhs.mil.

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that are capable of supporting antibody production by lupus B cells in vitro¹¹ such CD8 Th cells remain an uncommon finding compared to the large body of evidence demonstrating that CD4 T cells are necessary and sufficient for lupus development¹².

A useful model for studying the mechanistic role of CD4 T cells in lupus pathogenesis is the parent-into-F1 (P→F1) model of chronic graft-vs-host disease (GVHD). In this model, a lupus-like disease is induced in normal F1 mice following the transfer of parental strain CD4+ T cells¹³. Disease is a consequence of donor CD4 T cell recognition of allogeneic host MHC II molecules^{14,15} resulting in cognate delivery of CD4 help to potentially all host B cells¹⁶ resulting in polyclonal host B cell activation, the production of characteristic lupus-related autoantibodies and an immune complex glomerulonephritis^{17–19}. Because mouse T cells do not express MHC II, lupus-like GVHD can be induced in P→P transfers that involve solely an MHC II disparity e.g., B6→bm12 or vice versa²⁰ further emphasizing the critical role of donor CD4 T cells in initiating disease. Importantly, if parental CD8 T cells are administered in addition to CD4 T cells and the disparity between parent and F1 involves both MHC I and II, disease phenotype is converted from a lupus like chronic GVHD to an acute lethal disease in which donor CD8 T cells mature into CTL specific for host MHC I. The resulting attack on host immune and hematopoietic system results in a profound immunodeficiency that can be lethal^{21,22}. Thus, lupus-like disease in this model is thought to result solely from the activation of an oligoclonal population of alloreactive donor CD4 T cells specific for host MHC II and that concurrent activation of donor CD8 T cells qualitatively alters disease phenotype such that B cells are eliminated and lupus like disease is prevented²¹.

Autoantibodies characteristic of lupus are seen in a number of different P→F1 combinations²³ however only a few combinations result in sustained immune complex glomerulonephritis (ICGN) that resembles human lupus renal disease. A well studied combination is the transfer of DBA/2 lymphocytes into B6D2F1 mice (DBA→BDF1)^{17,18,24–26} and has also been reported to exhibit sex based differences i.e. greater renal disease severity is seen in females^{27–29}. Despite protocol variations in donor cell number or frequency of donor transfers, lupus-like disease is reliably induced in DBA→F1 mice following the transfer of unfractionated DBA splenocytes. Of note, the DBA donor inoculum contains both CD4 and CD8 T cells and the recipient has both an MHC I + II disparity, however maturation of donor DBA CD8 T cells into anti-host effector CTL is defective due in part to an approximate 10-fold reduction in the anti-F1 pCTL frequency compared to B6 mice³⁰ and preferential Th2 cytokine production by DBA CD4 T cells³¹. As a result, DBA→F1 mice develop chronic lupus-like GVHD rather than acute GVHD.

Because donor DBA CD8 T cells have not been shown to play a role in lupus-like disease expression much less in sex based differences in disease severity in DBA→F1 mice, we initially sought to examine potential sex based differences in donor CD4 Th cell help for B cells by formally demonstrating that donor CD8 T cells are irrelevant to disease expression. Surprisingly, we observed that DBA CD8 T cells play a critical role in sex based differences by exerting differential effects on host CD4 T cells, particularly ICOS^{hi} cells, and on IL-21 production.

Materials and Methods

Mice

6–8 week old male and female DBA/2J (DBA) (H-2^d) and B6D2F1 (BDF1)(H-2^{b/d}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures were pre-approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of Health Sciences.

Induction of GVHD

Single cell suspensions of DBA splenocytes were prepared as described³² and transferred into BDF1 hosts by tail vein injection. Donor and host mice were age and sex matched and male donors were transferred into male hosts (m→M) and female donors into female hosts (f→F). Prior to all transfers, CD4 and CD8 T cells were quantitated by flow cytometry. Recipient F1 mice received either: a) unfractionated splenocytes (CD8 intact→F1) containing 14×10^6 CD4 T cells and 4.6×10^6 CD8 T cells (approximately $80\text{--}90 \times 10^6$ total splenocytes); or b) splenocytes depleted of CD8 T cells (CD8 depleted→F1) containing 14×10^6 CD4 T cells. For CD8 depletion studies, CD8 T cells were positively selected and removed from the donor population using magnetic beads (Dynabeads, Mouse CD8, Lyt2) purchased from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. For CD8 depleted→F1 transfers, flow cytometry analysis confirmed that the donor inoculum contained <1% contaminating CD8⁺ T cells.

Flow cytometric analysis

Spleen cells were first incubated with anti-murine Fcγ receptor II/III mAb, 2.4G2 for 10 min and then stained with saturating concentrations of Alexa Fluor 488-conjugated, biotin-conjugated, PE-conjugated, APC-conjugated, PerCPCy5.5-conjugated, Pacific Blue-conjugated, or Pacific Orange-conjugated mAb against CD4, CD8, B220, H-2K^b, I-A^b, CD44, CD62L, ICOS (CD278), CD80, CD86, CD21, CD23, and CD138. All mAbs were purchased from either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), or Invitrogen (Carlsbad, CA). Biotinylated primary mAb were detected using either streptavidin-PE-Texas Red (BD Bioscience) or streptavidin-Alexa Fluor 700 (Invitrogen). Cells were fixed in 1% paraformaldehyde and multi-color flow cytometric analyses were performed using a BD FACScan flow cytometer or BD LCRII flow cytometer (BD Biosciences, San Jose, CA). Lymphocytes were gated by forward and side scatter and fluorescence data were collected for a minimum of 10,000 gated cells. Studies of donor T cells were performed on a minimum of 4,000 gated cells that were positive for CD4⁺ or CD8⁺ and negative for MHC class I of the uninjected parent.

Cytokine Expression by PCR

RNase-free plastic and water were used throughout the assay. Splenocytes (1×10^7) were homogenized in 1 ml of RNA-STAT-60 (Tel-Test, Friendswood, TX). RNA samples were reverse transcribed with Moloney murine leukemia virus reverse transcriptase according to manufacturer's protocol (Applied Biosystems International (ABI), Foster City, CA). All real time PCR primers (IFN-γ, IL-6, IL-21, and Mx-1) were purchased from Applied Biosystems.

Kidney Histopathology

Kidney tissue was fixed in 10% buffered formalin and processed for routine paraffin embedding and histological sectioning. Three-micron-thick sections, stained with periodic acid-Schiff stains, were blindly scored by a renal pathologist (MH). The severity of proliferative glomerulonephritis (GN score) and interstitial nephritis (IN score) were determined according to a previously described semi quantitative scoring system developed for a murine model of lupus nephritis³³. Briefly, the scoring for GN is: 1+ focal and segmental mesangial proliferative changes, 2+ diffuse mesangial proliferative changes, 3+ diffuse proliferative GN with focal crescents, 4+ diffuse proliferative GN with crescents in >50% of glomeruli. The scoring for IN is: 1+ patchy mononuclear cell interstitial infiltrate, 2+ diffuse mononuclear cell interstitial infiltrate, 3+ necrotizing interstitial infiltrates with edema and focal tubular atrophy, 4+ diffuse interstitial fibrosis and tubular atrophy.

Immunofluorescence Microscopy

Two micron-thick cryostat sections of unfixed kidney tissue were cut, allowed to air dry, and were fixed in cold acetone for 10 minutes. After rinsing twice with cold PBS, the sections were incubated for 30 minutes at room temperature in a humidified chamber with fluorescein isothiocyanate conjugated polyclonal antisera against mouse IgG1 (1:10 dilution in PBS) or IgG2a (1:20 dilution) (both from Southern Biotech, Birmingham, AL). Slides were then rinsed twice for 5 min each in PBS and coverslipped with Fluoromount. Immunofluorescence staining intensity in glomerular capillary walls and mesangium were blindly scored for each slide by a renal pathologist (MH), using a semi quantitative scale of 0 – 4, in increments of 0.5.

Electron Microscopy

Ultrastructural features were examined in glomeruli from 1–2 mice from each of the experimental groups. Formalin-fixed renal cortex was post-fixed in 3% glutaraldehyde and processed for electron microscopy using standard techniques as described previously³⁴. Ultrathin sections were viewed with a Philips CM10 electron microscope (Bothell, WA), and representative photographs were taken.

Urine Protein

Urine protein was quantitated by dipstick (Albustix, Bayer Pittsburgh, PA).

Serum chemistry

Serum obtained at the time of sacrifice (13 weeks) was tested for BUN, cholesterol, albumin and triglycerides using a VITROS 350 Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY) according to the manufacturer's protocol.

Serological studies

Mice were bled at the times indicated and sera tested by ELISA for the presence of IgG antibodies to ssDNA as described³⁵.

Statistical Analysis

Data were analyzed by unpaired Student's t test. Non-parametric data were analyzed by Mann-Whitney.

Results

Donor CD8 T cells are required for sex based differences in lupus-like renal disease severity in DBA→F1 mice

Given the central role of donor CD4 T cells in inducing lupus-like renal disease in the p→F1 model, we first sought to determine whether there were sex based differences in the ability of DBA CD4 T cells to provide help to F1 B cells. In these experiments we eliminated any contributory role for donor CD8 T cells by transferring DBA donor splenocytes depleted of CD8 T cells into age and sex matched cohorts composed of both m→M and f→F transfers (CD8 depleted→F1). The positive controls consisted of a second control cohort of age-matched m→M and f→F transfers receiving unfractionated DBA splenocytes (CD8 intact→F1). Both cohorts contained negative controls consisting of uninjected age and sex matched F1 mice. All donor inocula contained 14×10^6 CD4 T cells and the CD8 intact cohort also received 4.6×10^6 CD8 T cells for both sexes. By 13 weeks, clinical evidence of nephrotic syndrome was observed only in members of the CD8 intact f→F group and included ascites (3/5 mice) and grossly lipemic serum (5/5 mice). All mice were sacrificed at 13 weeks. Ascites and grossly

lipemic serum were not observed in CD8 intact m→M or either of the CD8 depleted→F1 groups.

Serum chemistries confirmed the clinical impression of nephrotic syndrome in that CD8 intact f→F mice exhibited significantly greater BUN (Fig. 1A), lower albumin (Fig. 1C) and greater triglycerides (Fig. 1E) compared to CD8 intact m→M or uninjected controls. Both CD8 intact m→M and f→F exhibited elevated significantly elevated cholesterol compared to uninjected control mice (Fig. 1G). CD8 depleted f→F mice exhibited significantly greater serum BUN (Fig. 1B) and surprisingly greater serum albumin (Fig. 1D) compared to m→M GVHD mice however depletion of donor CD8 T cells eliminated the sex based differences in triglycerides (Fig. 1F) and cholesterol (Fig. 1H) between m→M and f→F mice.

Sex based differences in renal disease severity are lost with donor CD8 T cell depletion

Kidneys were scored blindly for glomerular and tubulo-interstitial disease severity at the time of sacrifice (13 weeks) as described in Methods. For the CD8 intact→F1 cohort, sex based differences were seen for both glomerular (Fig. 2A) and interstitial scores (Fig. 2B) with f→F exhibiting significantly higher scores vs. m→M GVHD mice. Only 1/5 m→M mice exhibited glomerular disease (Glomerular score >1) vs. 5/5 f→F GVHD mice. Sex based differences in renal scores were lost in CD8 depleted →F1 mice due to a combination of worsening of some m→M scores and improvement in some f→F scores.

CD8 intact f→F GVHD mice exhibit severe membranous GN

To examine the pathologic basis of the clinical and serum chemistry results indicating severe nephrotic syndrome in CD8 intact f→F GVHD mice, immunofluorescent staining for deposited IgG1 and IgG2a was performed on tissue from both CD8 intact→F1 and CD8 depleted→F1 cohorts and severity of deposition quantitated as described in Methods and shown in Fig. 2C-F. Sex based differences are seen for CD8 intact→F1 mice but not for CD8 depleted→F1 mice. Specifically, CD8 intact f→F mice exhibited significantly greater glomerular capillary wall deposition of both IgG2a (Fig. 2C) and IgG1 (Fig. 2D) and these sex-based differences were lost for CD8 depleted→F1 mice. By contrast, reverse sex based differences were seen for mesangial deposition with CD8 intact m→M mice exhibiting significantly greater mesangial deposition of IgG2a compared to CD8 intact f→F mice (Fig. 2E). There were no significant differences in mesangial IgG1 deposition between these two groups (Fig. 2F). CD8 depleted→F1 mice did not exhibit sex based differences in IgG1 or IgG2a deposition in either the GCW or the mesangium.

Representative IF staining for IgG2a is shown in Fig. 3 and demonstrates little to no detectable deposition in either uninjected male (Fig. 3A) or female (Fig. 3B) control F1 mice. CD8 intact m→M mice exhibit a predominantly mesangial pattern with weak GCW staining (Fig. 3C) whereas CD8 intact f→F mice exhibit striking, confluent granular GCW staining in a typical membranous pattern, in most instances without mesangial staining (Fig. 3D). CD8 depleted m→M (Fig. 3E) and f→F mice (Fig. 3F) exhibit a mixture of mesangial and GCW staining, the latter being milder than in CD8 intact f→F mice.

Electron microscopy confirms the membranous pattern and also demonstrates more extensive membranous change in CD8 intact f→F mice (Fig. 4). A normal male F1 (Fig. 4A) demonstrates only very rare mesangial electron-dense deposits (EDD) (arrow). No subepithelial deposits are present and podocyte foot processes are generally intact. CD8 intact m→M (Fig. 4B) demonstrates expansion of mesangial matrix with numerous mesangial EDD consistent with the immunofluorescence findings for IgG2a deposition (Fig. 2E and Fig. 3C). There are multiple subepithelial deposits, although in a somewhat segmental distribution with absence of deposits in some portions of capillary loops. Very rare subendothelial deposits were

also noted (arrow), and there is extensive, almost complete podocyte foot process effacement. By contrast, CD8 intact f→F (Fig. 4C) shows numerous subepithelial deposits diffusely involving capillary loops, as well as a small number of mesangial deposits (arrow). Extensive, almost complete podocyte foot process effacement is also seen. Figs 4D & E are from mice receiving CD8 depleted donor inocula and demonstrate for m→M transfer (Fig. 4D) there is an expansion of mesangial areas with a smaller amount of mesangial EDD (arrows) than in CD8 intact m→M, again consistent with the immunofluorescence findings for IgG2a in this group (Fig. 2E). There are also many subepithelial deposits, although as with CD8 intact m→M there was an absence of deposits in some portions of capillary loops (arrowhead). Podocyte foot processes are diffusely effaced. Similarly, for CD8 depleted f→F (Fig. 4E) there is also mild expansion of the mesangial matrix with occasional mesangial EDD (arrow). There are numerous subepithelial deposits, albeit not quite as confluent as in CD8 intact f→F (Fig. 4C). Podocyte foot processes are diffusely effaced.

Taken together, the results of Figs 2 – 4 support the conclusion that all DBA→F1 mice exhibit MGN, but that the amounts of subepithelial and mesangial immune complex deposits are altered by the sex of the P→F1 combinations and the presence or absence of CD8 T cells in the donor inoculum. The most severe deposition of Ig, as seen in the GCW on IF and as subepithelial deposits on EM, are seen in CD8 intact f→F mice.

More severe renal disease in CD8 intact f→F GVHD mice is preceded by greater proteinuria and serum anti-ssDNA ab

The kinetics of proteinuria over the 13 week course are shown in Fig. 5 for both cohorts. For CD8 intact→F1 mice (Fig. 5A), both m→M and f→F mice shown an initial mild increase in proteinuria peaking during weeks 2–4 after which the two groups diverge with proteinuria increasing from weeks 6–10 for CD8 intact f→F mice and declining during this same period in CD8 intact m→M mice. The differences in proteinuria become significant at weeks 8 and 10. Sex based differences are not seen in CD8 depleted→F1 mice (Fig. 5B) with both m→M and f→F mice exhibiting similar severity levels and both groups exhibit a similar peak at 8 weeks.

Anti-ssDNA ab are a useful marker of non-specific autoreactive B cell activation in this model³⁵ and also demonstrate sex based differences in CD8 intact DBA→F1 mice that are lost in CD8 depleted DBA→F1. As shown in Fig. 5C, CD8 intact f→F GVHD mice exhibit a striking increase in anti-ssDNA ab with a peak at 4 weeks that is significantly greater than that of CD8 intact m→M GVHD mice (f→F vs. m→M = 3-fold at 6 weeks). Sex based differences are not seen for CD8 depleted→F1 mice (Fig. 5D) due to a striking reduction in f→F anti-ssDNA values rather than an elevation of m→M values. These results indicate that donor CD8 T cells are critical for the striking increase in B cell activation seen for f→F vs m→M mice.

Sex based differences in F1 host B cell numbers and activation are seen in the CD8 intact →F1 cohort and lost in the CD8 depleted→F1 cohort

To determine whether the greater autoantibody production in CD8 intact f→F vs. m→M mice was accompanied by changes in T cell and B cell parameters, the spleens of both cohorts were examined at 13 weeks at the time of sacrifice for lymphocyte populations. As shown in Fig. 6, sex based differences were observed in the CD8 intact cohort such that f→F mice exhibited significantly greater numbers of total B cells (Fig. 6A, greater B cell upregulation of MHC II (Fig. 6B) and greater numbers of both follicular B cells (6C) and plasma cells (6E) compared to m→M mice. These sex based differences are not seen in the CD8 depleted→F1 cohort due to a combination of a reduction in f→F values and an increase in m→M values. In contrast, marginal zone (MZ) B cells were reduced in all p→F1 groups vs. sex-matched uninjected controls (Fig. 6D). Although sex based were seen for both CD8 intact→F1 and CD8 depleted→

F1 mice there was a reversal such that MZ B cell numbers were significantly greater in m→M vs. f→F. With the exception of MZ B cells, all B cell parameters and plasma cell numbers in Fig. 6 are also elevated over control F1 for both sexes in the CD8 depleted→F1 mice consistent with active renal disease shown in Figs. 2–4.

Donor CD8 T cells are associated with alterations in host CD4 Th subsets, particularly T follicular helper (Tfh) cells

CD4 T cells play a critical role in promoting B cell autoantibody production both in human lupus^{1,10,36} and in the p→F model of lupus^{15,16}. CD4 Th cells can be divided into naïve and activated subsets based on their expression of CD44 (hi and low respectively) and CD44^{hi} CD4 T cells further subdivided with combined CD62L expression such that CD44^{hi}, CD62L^{low} CD4 T cells are composed of activated effector cells and CD44^{hi}, CD62L^{hi} are composed of central memory cells³⁷. Importantly, upregulation of ICOS designates the T-follicular helper (Tfh) subset which has been shown to be critical in providing help to B cells and promoting Ig production, in large part through IL-21^{38–40}. The numbers of these CD4 T cell subsets are shown in Fig. 7 and demonstrate sex based differences for the CD8 intact→F1 cohort in both donor (Fig. 7A, B) and host (Fig. 7C,D) CD4 T cell subsets. For example, CD8 intact f→F mice exhibit significantly greater numbers of donor ICOS^{hi} (Fig. 7A), total donor CD44^{hi} CD4 T cells (Fig. 7A), donor effector CD4 T cells (CD44^{hi}, CD62L^{lo}) (Fig. 7B) and donor central memory CD4 T cells (CD44^{hi}, CD62L^{hi}) (Fig. 7B) compared to CD8 intact m→M mice. These sex based changes are preserved in the CD8 depleted→F1 cohort with f→F mice exhibiting significantly greater numbers of all four cell populations compared to CD8 depleted m→M mice. Additionally, for f→F mice there are no significant differences in any given donor CD4 T cell subsets when comparing CD8 intact→F1 vs CD depleted→F1 mice.

As with donor CD4 T cells, CD8 intact f→F mice exhibit significantly greater numbers of all host CD4 T cell subsets compared to CD8 intact m→M mice i.e host ICOS^{hi} (Fig. 7C), CD44^{hi} (Fig. 7C), effector helper (CD44^{hi}, CD62L^{lo}) (Fig. 7D) and central memory (CD44^{hi}, CD62L^{hi}) CD4 T cells (Fig. 7D) are all significantly increased in f→F vs m→M mice. However in contrast to CD8 intact→F1 mice, donor CD8 depletion had a different effect on host CD4 subsets in that sex based differences in host CD4 effector helper T cells (CD44^{hi}, CD62L^{lo}) are lost in the CD8 depleted cohort. Additionally, the total number of host CD4 Tfh for CD8 depleted f→F mice is significantly reduced compared to CD8 intact f→F mice. Despite the reduction in Tfh cells for CD8 depleted f→F mice, Tfh cells are still significantly greater compared to CD8 depleted m→M mice. CD8 depleted f→F mice also exhibited reductions in total CD44^{hi} and effector populations compared to CD8 intact f→F mice however these differences were not significant. Thus, the major effect of donor CD8 T cell depletion is a reduction in the absolute numbers of host, not donor, CD4 Tfh cells and a loss of female skewing of CD4 effector (CD44^{hi}, CD62L^{low}) cells.

Sex based differences in IL-21 gene expression are seen in CD8 intact →F1 cohort and reduced in CD8 depleted f→F mice

ICOS-dependent Tfh cells play an important role in promoting B cell maturation and IgG production in part through production of IL-21^{38,41,42}. Importantly, IL-21 has been also shown to be important in the production of IgG autoantibodies in spontaneous murine lupus⁴³. To further address a possible role for Tfh cells in the DBA→F1 model of lupus, we sought evidence for sex based differences in IL-21 by measuring IL-21 gene expression by unfractionated F1 splenocytes at 13 weeks. For CD8 intact→F1 mice, f→F mice exhibit striking elevations (>60 fold) of IL-21 gene expression over sex matched uninjected control F1 mice (Fig. 8A). Moreover, these values are approximately 4 fold greater than those seen in CD8 intact m→M mice. By contrast, CD8 depleted f→F mice exhibit IL-21 gene expression that is significantly reduced compared to CD8 intact f→F mice however it is still significantly greater than that for

CD8 depleted m→M mice. No significant sex based differences were seen for IFN- α inducible gene mx-1 (8B). Interestingly, there was significantly greater, IFN- γ (Fig. 8C) and IL-6 (Fig. 8D) gene expression for CD8 intact m→M vs f→F mice although the elevations were low level. The sex based differences were lost in the CD8 depleted cohort. Importantly, the striking elevations of IL-21 gene expression with female skewing is not seen for other cytokines important in T and B cell responses, thereby underscoring the importance of IL-21 in disease expression.

Discussion

Our results make two novel observations regarding the pathogenesis of murine lupus-like renal disease. Firstly, sex based differences (i.e. greater female severity) in clinical and histological severity of GN are critically dependent on the presence of CD8 T cells in the donor inoculum. The critical role of donor CD8 T cells was directly demonstrated through depletion of CD8 T cells from the donor inoculum prior to transfer which resulted in the loss of sex based differences in renal disease severity. Because donor CD4 T cells are central to disease pathogenesis in this model, selective depletion of donor CD8 T cells does not block donor CD4 T cell driven lupus-like renal disease development but instead attenuates the female skewing of severity. A second novel observation is that changes in host CD4 T cells, particularly the ICOS^{hi} Tfh cell subset, are associated with sex based differences in disease severity, possibly through IL-21 production. Here, the role of host CD4 Tfh cells was shown by flow cytometry studies demonstrating that this subset was the only donor or host CD4 T cell subset that was significantly reduced long term in mice receiving CD8 depleted donor cells. Further supporting a role for host CD4 T cells in mediating sex based differences in disease severity was a loss of sex based differences in host CD4 effectors (CD44^{hi}, CD62L^{lo}) seen in CD8 depleted→F1 mice. Thus, greater severity of renal disease seen for females in CD8 intact →F1 mice is accompanied by greater numbers of donor and host CD4 effector and CD4 ICOS^{hi} Tfh cells in females. With depletion of donor CD8 T cells, there is a loss of sex based differences in renal disease that is accompanied by changes in the host but not donor CD4 effectors and Tfh cells. Supporting evidence for a role for Tfh cells was provided by splenic IL-21 gene expression, a product of Tfh cells that promotes IgG antibodies to include autoantibodies ³⁹. IL-21 gene expression was strikingly increased in f→F compared to m→M mice for the CD8 intact→F1 cohort and these sex based differences were significantly reduced but not totally eliminated in the CD8 depleted→F1 cohort.

It is well established that donor CD4 T cells are necessary and sufficient for lupus-like disease induction ^{14,16,20}. By contrast, donor CD8 T cells are not required for lupus like chronic GVHD and instead, their inclusion in the donor inoculum typically converts lupus like chronic GVHD to acute GVHD ³⁰. Donor CD8 T cells have a well studied role in mediating acute GVHD following their maturation into effector CTL specific for host MHC I resulting in elimination of host lymphocytes, particularly B cells (reviewed in ⁴⁵). By approximately day 12–14 after transfer, donor CD8 CTL effectors undergo homeostatic contraction leaving donor CD4 T cell help for host B cells unopposed ³⁵. Thus, incomplete elimination of host splenocytes at the time of donor CD8 contraction, as seen following the transfer of donor cells with defective CD8 CTL function (e.g. perforin defective donor CD8 T cells) allows re-expansion of host cells, particularly B cells due to the continued presence of donor CD4 T cells capable of providing continued T-B collaboration and the transition to a lupus-like phenotype ⁴⁶. DBA CD8 T cells exhibit defective in vivo anti-host CTL maturation due to a reduced precursor CTL frequency for F1 MHC alloantigens ³⁰ and a Th2 skewing of DBA CD4 cells ³¹. As a result, in vivo CD8 CTL development is markedly attenuated in DBA→F1 mice, elimination of host B cells is incomplete and mice develop lupus rather than acute GVHD as donor CD8 T cells contract. Our results indicate that despite the failure of DBA CD8 T cells to mature into CTL effectors capable of completely eliminating host B cells, their sub-optimal activation and

down regulation during the first two weeks after transfer is likely a necessary prerequisite for sex based differences in this model. We have recently observed that sex based differences in donor CD8 T cell survival in DBA→F1 mice influences donor CD4 T cell engraftment (ms in preparation).

Sex based differences in the above mentioned CD4 T cell subsets are associated with sex based differences in B cell subsets. Specifically, greater numbers of donor and host CD4 Tfh cells seen for CD8 intact f→F vs m→M mice are associated in turn with greater numbers of total B cells, greater B cell activation (MHC II upregulation), greater numbers of follicular B cells and plasma cells, greater serum autoantibody production and greater proteinuria, all of which support the idea that CD8 intact f→F mice exhibit greater CD4 T cell driven B cell autoantibody production resulting in more severe renal disease. These sex based differences in B cell numbers, serum autoantibody levels and proteinuria are all lost in CD8 depleted →F1 mice and are associated with a reduction in the numbers of host Tfh cells in females and a loss of sex based differences in host CD4 memory T cells supporting a potentially important role for these CD4 T cell subsets in mediating sex based differences in autoantibody production and renal disease severity.

The foregoing results raise the possibility that in addition to the obligatory role of donor CD4 T cells in lupus induction, host CD4 T cells may play a non-obligatory role that shapes disease expression and severity. Because disease in this model is initiated and perpetuated by donor CD4 T cells, participation of host CD4 T cells in disease expression supports the idea that once tolerance is broken, the pool of (auto) ag-specific CD4 T cells can be expanded, possibly through epitope spreading and clonal expansion as described⁴⁹ to exacerbate disease expression and the production of pathogenic autoantibodies. Our results are consistent with the novel possibility that CD8 T cells are an important contributor to CD4 T cell clonal expansion and epitope spreading characteristic of active lupus and support further studies of the role of CD8 T cells in shaping sex based differences in lupus expression.

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Figure 1. Sex based differences in serum chemistries in DBA→F1 mice using unfractionated donor splenocytes are reduced using CD8 T depleted donor cells

Male and female BDF1 mice received sex matched unfractionated DBA splenocytes (CD8 intact→F1)(A, C, E, G) or DBA splenocytes selectively depleted of CD8 T cells (CD8 depleted→F1)(B, D, F, H) as described in Methods (n=5 for all four experimental groups). Both cohorts were followed for 13 weeks at which time sera was analyzed for serum BUN (A,B) albumin (C, D), triglycerides (E,F), and cholesterol (G,H). Values represent individual mice with group means shown as bars. Values from age and sex matched uninjected control male (M)(n=4) and female (F)(n=5) F1 mice are shown for comparison. For all figures: * p <0.05, ** p<0.01, ***p <0.005

Figure 2. Greater renal disease severity is seen in CD8 intact f→F vs. m→M mice and is lost in CD8 depleted f→F mice

Kidneys from both cohorts were processed at 13 weeks as described in Methods and scored blindly for: A) proliferative glomerulonephritis (GN) score, B) interstitial nephritis (IN) score, glomerular capillary wall (GCW) deposition of IgG2a (C) or IgG1 (D) and mesangial deposition IgG2a (E) or IgG1 (F). Immunofluorescence staining intensity in both glomerular capillary walls and mesangium was graded on a 0–4 scale in increments of 0.5. Values represent scores for individual mice.

Figure 3. Representative immunofluorescence micrographs of glomerular deposition of IgG2a
 Experimental protocol is as outlined in Figs. 1 and 2 and IgG2a IF staining shown for: A) normal male; B) normal female; C) CD8 intact m→M; D) CD8 intact f→F; E) CD8 depleted m→M and F) CD8 depleted f→F. Normal mice showed no more than trace (0.5 intensity score) mesangial staining for IgG2a, with no capillary wall staining. Patterns of staining in each of the experimental groups are discussed in the text. Original magnification of each photomicrograph is $\times 400$.

Figure 4. Electron microscopy of representative glomeruli from normal and experimental mice
 Experimental groups are as described in Fig. 1 and EMs were performed as described in Methods. A) normal male F1: arrow denotes rare mesangial deposits. (original magnification $\times 6300$). B) CD8 intact m $\rightarrow$ M: numerous mesangial deposits and multiple subepithelial deposits are seen although in a somewhat segmental distribution with absence of deposit in small portions of the capillary loop. Very rare subendothelial deposits were noted (arrow) (original magnification $\times 8000$). C) CD8 intact f $\rightarrow$ F: diffuse, confluent subepithelial deposits, and occasional mesangial deposits (arrows) are seen (original magnification $\times 6300$). D) CD8 depleted m $\rightarrow$ M: a smaller number of mesangial deposits (bottom two arrows) are seen compared to CD8 intact m $\rightarrow$ M mice and multiple subepithelial deposits are seen though with absence of deposit segmentally along the capillary loop (top arrow) (original magnification $\times 6300$). E) CD8 depleted f $\rightarrow$ F: findings are similar to those in CD8 intact f $\rightarrow$ F with occasional mesangial deposits (arrows) and many subepithelial deposits, although the latter are somewhat less confluent than in intact f $\rightarrow$ F (original magnification $\times 6300$).

Figure 5. Greater severity of proteinuria and serum anti-ssDNA levels seen for CD8 intact f→F vs m→M mice is lost with donor cell CD8 depletion

Experimental groups are as in Fig. 1. Mice were tested at the indicated time points for proteinuria (A, B) and serum anti-ssDNA ab (C, D) as described in Methods for F1 cohorts receiving CD8 intact (A, C) and CD8 depleted (B, D) donor cells. Values represent group mean $\pm$ SEM.

Figure 6. CD8 intact f→F mice have increased numbers of B cells as well as increased B cell activity that is lost with donor cell CD8 depletion

Experimental groups are as in Fig. 1. Total splenocytes were stained for analysis by flow cytometry as described in Methods for: A) B cells (B220⁺), B) MHC II expression on B cells (expressed as percent increase in mean fluorescence intensity relative to sex matched F1 control); C) follicular B Cells (B220⁺CD21^{Int}CD23^{Hi}); D) marginal zone B cells (B220⁺CD21^{Hi}CD23^{Lo}); E) and plasma cells (B220⁺CD138^{Hi}). Values represent group mean $\pm$ SEM.

Figure 7. Donor CD8 depletion results in a quantitative reduction in host but not donor T follicular helper cells in both f→F and m→M mice

Experimental groups are as in Fig. 1. Total splenocytes were stained for analysis by flow cytometry as described in Methods. CD4 subset analysis is shown for donor (A,B) and host (C, D) T cells. Subsets are shown for: A) donor CD4⁺ICOS^{Hi} T cells and donor CD4⁺CD44^{Hi} T cells, B) donor CD4⁺CD44^{Hi}CD62L^{Lo}, and donor CD4⁺CD44^{Hi}CD62L^{Hi} T cells, C) host CD4⁺ICOS^{Hi} T cells and host CD44^{Hi} T cells, and D) host CD44^{Hi}CD62L^{Lo} and host CD4⁺CD44^{Hi}CD62L^{Hi} T cells. Values represent group mean ± SEM.

Figure 8. Sex based differences in IL-21 gene expression in CD8 intact f→F mice are reduced but not lost in CD8 depleted→ F1 mice

Experimental protocol is as in Fig. 1. Splenic mRNA was isolated and cytokine gene expression performed as described in Methods. Results are shown fold increase over uninjected sex matched control F1 mice (group mean $\pm$ SEM) for: A) IL-21, B) Mx-1, C) IFN-g and D) IL-6.