CD8 T cells contribute to lacrimal gland pathology in the nonobese diabetic mouse model of Sjögren syndrome

Jennifer Y Barr1, Xiaofang Wang1, David K Meyerholz2 and Scott M Lieberman1,3

Sjögren syndrome is an autoimmune disease characterized by targeted destruction of the lacrimal and salivary glands resulting in symptoms of severe ocular and oral dryness. Despite its prevalence, the mechanisms driving autoimmune manifestations are unclear. In patients and in the nonobese diabetic (NOD) mouse model of Sjögren syndrome, lymphocytic infiltrates consist of CD4 and CD8 T cells, although the role of CD8 T cells in disease pathogenesis has been largely unexplored. Here, we evaluated the contribution of CD8 T cells to lacrimal and salivary gland autoimmunity. Within the lacrimal and salivary glands of NOD mice, CD8 T cells were proliferating, expressed an activated phenotype, and produced inflammatory cytokines. Transfer of purified CD8 T cells isolated from the cervical lymph nodes (LNs) of NOD mice into NOD-severe combined immunodeficiency recipients resulted in inflammation of the lacrimal glands, but was not sufficient to cause inflammation of the salivary glands. Lacrimal gland-infiltrating CD8 T cells displayed a cytotoxic phenotype, and epithelial cell damage in the lacrimal glands was observed in recipients of CD8 T cells regardless of the presence of CD4 T cells. Collectively, our results demonstrate that CD8 T cells have a pathogenic role in lacrimal gland autoimmunity. The gland-specific pathogenicity of CD8 T cells makes them a valuable resource to further understand the mechanisms that discriminate lacrimal versus salivary gland autoimmunity and for the development of new therapeutics that target the early stages of disease.

RESULTS
Activated CD8 T cells are present in lacrimal and salivary gland infiltrates
To determine the role of CD8 T cells in lacrimal and salivary gland autoimmunity, we characterized CD8 T cells isolated from the lacrimal and salivary glands of male and female NOD mice, respectively. As demonstrated previously,12 the frequencies of CD4 and CD8 T cells were similar in infiltrates from lacrimal and salivary glands, with CD4 T cells constituting the majority of T cells in each gland, but with a population of CD8 T cells always present (Figure 1a). We assessed these CD8 T cells in gland infiltrates for the expression of surface activation markers and production of cytokines (Figure 1b).
This included analysis of the integrin CD11a, which has a role in T-cell costimulation and is upregulated on CD8 T cells after antigen stimulation, thus serving as a surrogate marker for T-cell activation. We also examined cell surface expression of CD69, which is upregulated as a result of T-cell stimulation through the T-cell receptor, or in the presence of inflammatory signals (e.g., interferon-α/β (IFN-α/β)). In both lacrimal and salivary glands, the majority of CD8 T cells expressed CD11a, while a large subset also expressed CD69 (Figure 1b). A subset of CD8 T cells isolated from the glands was actively proliferating based on Ki67 staining (Figure 1b). The majority of CD8 T cells produced IFNγ upon stimulation, and a subset of these coproduced tumor necrosis factor-α (Figure 1b). Similarly, lacrimal and salivary gland-infiltrating CD4 T cells expressed an activated phenotype, were proliferating and produced IFNγ and tumor necrosis factor-α following stimulation (Figure 1c). Thus, the majority of CD8 and CD4 T cells in lacrimal and salivary gland infiltrates displayed an activated phenotype and were capable of producing inflammatory cytokines.

**CD8 T cells promote dacryoadenitis, but not sialadenitis**

To determine the pathogenicity of CD8 T cells in the context of lacrimal and salivary gland autoimmunity, we tested their ability to promote the development of dacryoadenitis or sialadenitis. To address this question, we used our previously established adoptive transfer model of Sjögren syndrome in which the transfer of cervical lymph node (LN) cells into sex-matched recipients results in gland inflammation similar to that observed in spontaneous disease (and manuscript in preparation). CD8 T cells were purified (>96% of total cells and >98% of T cells) from cervical LN of male or female NOD mice and transferred into sex-matched NOD-severe combined immunodeficiency (NOD-SCID) recipients (Figure 2a). As a comparison, we transferred purified CD8 T cells that were recombined with the CD8-depleted population (non-CD8+CD8) into sex-matched NOD-SCID recipient mice. Five weeks after transfer, the degree of dacryoadenitis or sialadenitis was assessed. In male recipients, a similar level of dacryoadenitis developed in mice that received CD8-only cells relative to mice that received non-CD8+CD8 cells, with some mice in the CD8-only group developing diffuse inflammation (Figure 2b and Supplementary Figure 1a). While low levels of sialadenitis were observed in some recipients of non-CD8+CD8 cells, sialadenitis was not observed in male recipients of CD8-only cells (Figure 2b and Supplementary Figure 1b). In the NOD mouse model, male mice spontaneously develop dacryoadenitis, whereas a greater degree of sialadenitis develops in female NOD mice. Therefore, we determined if CD8 T cells could promote sialadenitis in female mice. Strikingly, while female recipients of non-CD8+CD8 cells developed sialadenitis, we did not detect sialadenitis in any female NOD-SCID recipients of CD8-only cells, despite the fact that some of these recipients developed dacryoadenitis (Figure 2c and Supplementary Figures 1c and d). Collectively, these data suggest that CD8 T cells can independently promote the development of dacryoadenitis in male NOD mice but not sialadenitis in female NOD mice.

Flow cytometric analyses of cervical LN cells in recipient mice 5 weeks after transfer confirmed that recipients of CD8-only cells had proportionally more CD8 T cells in the cervical LN relative to mice that received non-CD8+CD8 cells (Figure 2d). An outgrowth of CD4 T cells was observed in CD8-only recipients, yet this percentage was still significantly reduced relative to the percentage of CD4 T cells present in recipients of non-CD8+CD8 cells (Figure 2d). The outgrowth of CD4 T cells in the cervical LN of CD8-only recipients included a subset of CD4+Foxp3+ T-regulatory cells (Tregs).

**Figure 1** Gland-infiltrating CD8 T cells display an activated phenotype. (a) Representative flow cytometry plots of gland-infiltrating T cells from male lacrimal (top) or female salivary (bottom) glands from 12–17-week-old NOD mice. Plots were gated on live, CD3ε+ singlets. Numbers represent the frequency of cells in indicated gate. Graphs depict cumulative quantification of data from at least two independent experiments from male lacrimal (top) or female salivary (bottom) glands, n=7 per group. Each data point indicates an individual mouse and lines represent the mean. (b) Flow cytometric analyses of gland-infiltrating CD8 T cells from male lacrimal (left) or female salivary (right) glands from mice in a. Plots were gated on live, CD3ε+CD8ε+CD4ε+ singlets. Numbers represent the frequency of cells in each gate. (c) Flow cytometric analyses of gland-infiltrating CD4 T cells from male lacrimal (left) or female salivary (right) glands from mice in a. Plots were gated on live, CD3ε+CD8ε+CD4ε-Foxp3ε+ singlets. Numbers represent the frequency of cells in each gate. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.
(Supplementary Figure 2a). Corresponding to the overall reduction in the percentage of CD4 T cells in CD8-only recipients, the percentage of Tregs within the total T-cell population was also significantly reduced relative to recipients of non-CD8+CD8 cells (Supplementary Figure 2a). However, within the CD4 T-cell population the percentage of Tregs was similar in CD8-only recipients relative to recipients of non-CD8+CD8 cells (Supplementary Figure 2b), suggesting that the outgrowth of CD4 T cells in the cervical LN is equivalent for non-Tregs and Tregs. Despite the outgrowth of CD4 T cells in recipients of CD8-only cells, very few B cells were present, whereas recipients of non-CD8+CD8 cells had an appreciable proportion of B cells (Supplementary Figure 2c).

Analysis of cell composition in the lacrimal glands similarly showed that CD8 T cells comprised the major cell population in CD8-only recipients, yet the outgrowth of CD4 T cells noted in the cervical LN was also detected within these lacrimal gland infiltrates (Figure 2e). In recipients of non-CD8+CD8 cells, CD4 T cells constituted the majority of cells in the lacrimal glands, whereas CD8 T cells comprised a small subset of the population (Figure 2e), similar to the composition observed in spontaneous disease (Figure 1). Notably, the ratio of CD4:CD8 T cells was elevated within the lacrimal glands relative to the cervical LN in mice that received non-CD8+CD8 cells (Figure 2f), indicating a selectivity for CD4 T cells in the lacrimal glands, similar to that observed when dacryoadenitis develops spontaneously (Figure 2g). Although not significant, a similar difference was also observed in recipients of CD8-only cells (Figure 2f).

Whether, however, this reflects differential trafficking of CD4 T cells to the lacrimal glands or differential expansion of these cells within the lacrimal glands remains to be determined. Within the total T-cell population in the lacrimal glands, CD4+Foxp3+ Tregs were also significantly reduced in CD8-only recipients relative to recipients of non-CD8+CD8 cells (Supplementary Figure 2d). While not reaching statistical significance, CD4+Foxp3+ Tregs were reduced within the CD4+ population in CD8-only recipients relative to recipients of non-CD8+CD8 cells (Supplementary Figure 2e). This is in contrast to the cervical LN (Supplementary Figure 2b), although it is unclear if this is due to differences in the trafficking of CD4+Foxp3+ cells to the lacrimal glands or their expansion within the glands.

**Dacryoadenitis occurs in the absence of CD8 T cells**

Despite the presence of activated CD8 T cells in lacrimal gland infiltrates, the outgrowth of CD4 T cells in our CD8-only transfers and selective accumulation of CD4 T cells within lacrimal glands of NOD mice and NOD-SCID recipients suggested CD4 T cells have a key role in the development of dacryoadenitis. To determine if cervical LN cells could mediate lacrimal gland disease in the absence of CD8 T cells, we transferred CD8-depleted cervical LN cells from female NOD donors either alone (non-CD8) or with CD8 T cells added back (non-CD8+CD8) to sex-matched, NOD-SCID recipient mice. A similar degree of dacryoadenitis developed in recipients of non-CD8 cells and recipients of non-CD8+CD8 cells (Figure 3a). In contrast to the outgrowth of CD4 T cells observed in CD8-only transfers (Figures 2d–f), very few (<1%) CD8 T cells were detected in the cervical LN or lacrimal glands of recipients of non-CD8 cells (Figures 3b and c). Analysis of other lymphocyte populations demonstrated that both CD4 T cells and B cells were present, with no difference in the percentage of B cells observed between the two groups (Figures 3b and c and Supplementary Figure 3). Thus, CD8-depleted cervical LN cells can transfer dacryoadenitis.

**CD8 and CD4 T cells each contribute to dacryoadenitis**

We next assessed the level of spontaneous dacryoadenitis in NOD mice that were deficient for CD8 expression (CD8null) or CD4 expression (CD4null) and thus lacked CD8 or CD4 T cells, respectively. In contrast to our transfer model, the absence of CD8 T cells resulted in a significant decrease in dacryoadenitis in 11–14-week-old CD8null mice relative to age-matched wild-type (WT) NOD mice (Figure 4). Dacryoadenitis was also significantly reduced in CD4null mice relative to WT controls, while no difference in inflammation was observed between CD4null and CD8null mice (Figure 4). In contrast, 20–24-week-old CD8null mice developed increased dacryoadenitis relative to age-matched CD4null mice, which continued to display decreased dacryoadenitis compared with age-matched WT mice (Figure 4). Further, the degree of inflammation in old CD8null mice was increased relative to young CD8null mice, whereas no change was observed between young and old CD4null mice (Figure 4). For all genotypes, both T and B cells were present in the cervical LN and, as expected, CD8 or CD4 T cells were absent from the cervical LN of CD8null or CD4null mice, respectively (Supplementary Figure 4).

Collectively, these findings suggest that whereas CD8 T cells do not compensate for the lack of CD4 T cells, over time CD4 T cells can compensate for the lack of CD8 T cells in mediating dacryoadenitis. Although the degree of dacryoadenitis in 11–14-week-old CD8null and CD4null mice was reduced relative to WT NOD mice, some inflammation was still observed (i.e., focus score > 0), which is in contrast to mouse strains that are prone to lacrimal gland autoimmunity. Specifically, no foci (i.e., focus score = 0) were observed in lacrimal glands of 13-week-old male Balb/c mice (n = 5, data not shown). These data indicate that CD8 and CD4 T cells are independently capable of mediating some degree of lacrimal gland inflammation in NOD mouse strains. Collectively, the reduction in disease severity in the absence of either CD8 or CD4 T cells suggests that both cell types contribute to the early events that promote spontaneous dacryoadenitis.

**CD4 T cells are not required for CD8 T-cell-mediated dacryoadenitis**

Although the development of some dacryoadenitis in CD4null mice suggests inflammation in this context is mediated by CD8 T cells, the presence of CD4CD8 TCRαβ+ T cells with characteristics of CD4 T cells have been reported in CD4null mice.46 Thus, we cannot rule out the potential contribution of these cells to the dacryoadenitis observed in CD4null mice. Additionally, the consistent outgrowth of CD4 T cells in our CD8-only transfers (Figures 2d–f) suggested the possibility that CD8 T cells only cause dacryoadenitis in the presence of CD4 T cells. To address this, we transferred purified populations of fluorescence-activated cell-sorted CD8 T cells from male NOD donors (Figure 5a) into sex-matched, NOD-SCID recipients that were treated weekly with anti-CD4 or isotype control antibody. Similar to the outgrowth of CD4 T cells observed in our prior CD8-only transfers, an outgrowth of CD4 T cells was detected at 3 and 5 weeks post transfer in the peripheral blood of recipient mice treated with an isotype control antibody (Figure 5b). In contrast, CD4 T cells were undetectable in the blood of mice receiving the anti-CD4 antibody treatment (Figure 5b), indicating efficient depletion of CD4 T cells. Despite these differences, the degree of dacryoadenitis was similar in mice treated with anti-CD4 antibody relative to those treated with isotype control antibody (Figure 5c). The overwhelming majority of cells recovered from the cervical LN or lacrimal glands of anti-CD4 antibody-treated recipients were CD8 T cells, with <0.6% being CD4 T cells (Figures 5d and e).
Importantly, while the CD4:CD8 ratio again demonstrated an increase in CD4 T cells in the lacrimal glands relative to the cervical LN of isotype antibody-treated recipients, no such accumulation was noted in the lacrimal glands of anti-CD4 antibody-treated recipients (Figure 5f). Thus, in the absence of an outgrowth of CD4 T cells, CD8 T cells can independently infiltrate the lacrimal glands and promote the development of dacryoadenitis in our transfer model of Sjögren syndrome.

CD8 T cells promote lacrimal gland autoimmunity

JY Barr et al

Figure 2 Transfer of CD8 T cells causes dacryoadenitis, but not sialadenitis, in NOD-SCID recipients. (a) Representative plots to demonstrate CD8 T cells in pre- and postsort samples. Bulk cervical LN cells from male NOD mice were FACSorted into purified CD8 cells (CD8-only) or CD8-depleted cells (non-CD8). Plots were gated on singlets. Numbers represent the frequency of cells in indicated gate. (b) Quantification of lacrimal (left) and salivary (right) gland inflammation in male NOD-SCID recipients of male NOD donor cells. Recipients received CD8-depleted cells with CD8 T cells added back (non-CD8+CD8: n=8) or purified CD8 T cells alone (CD8-only: n=10). Data are pooled from at least two independent experiments. Symbols represent individual mice, lines are medians. Boxed symbols represent diffuse inflammation in which individual foci coalesced and could not be accurately enumerated. P-values were determined by Mann–Whitney U-test. (c) Quantification of salivary (left) and lacrimal (right) gland inflammation in female NOD-SCID recipients of female NOD donor cells. Recipients received non-CD8+CD8: n=10 or CD8-only: n=12. Data are pooled from two independent experiments. Symbols represent individual mice, lines are medians. P-values were determined by Mann–Whitney U-test. (d, e) Representative flow cytometry plots of cervical LN cells (d) or lacrimal gland-infiltrating cells (e) from male recipients represented in b. Plots were gated on live, CD3ε+ singlets. Numbers represent the frequency of cells in indicated gate. Graphs depict cumulative quantification of data pooled from two independent experiments (non-CD8+CD8: n=8; CD8-only: n=10) (d) or one experiment (non-CD8+CD8: n=4; CD8-only: n=8) (e). Each symbol represents an individual mouse, lines are means. P-values were determined by unpaired Student’s t-test. (f) Ratio of CD4 to CD8 T cells (log-transformed) present in the cervical LN or lacrimal glands of male recipients from (d, e). Each symbol represents an individual mouse, lines are means. P-values were determined by unpaired Student’s t-test. A full color version of this figure is available at the Immunology and Cell Biology journal online.

CD8 T cells mediate epithelial cell destruction

The presence of activated, inflammatory cytokine-producing CD8 T cells in the lacrimal glands (Figure 1) and the ability of CD8 T cells to promote the development of dacryoadenitis even in the absence of CD4 T cells (Figure 5) suggest that CD8 T cells have a pathogenic role in lacrimal gland autoimmunity. Upon activation, CD8 T cells differentiate into cytotoxic T cells and kill target cells when their cognate antigen is recognized.29 To confirm that CD8 T cells in the
CD8 T cells promote lacrimal gland autoimmunity

JY Barr et al

Figure 3 Lacrimal gland inflammation occurs in the absence of CD8 T cells. (a) Quantification of lacrimal gland inflammation in male NOD-SCID recipients of sorted male NOD donor cervical LN cells. Recipients received CD8-depleted cells with CD8 T cells added back (non-CD8+CD8; n = 13) or CD8-depleted cells alone (non-CD8; n = 19). Data are pooled from three independent experiments. Symbols represent individual mice, lines are medians. P-values were determined by Mann-Whitney U-test. (b) Representative flow cytometry plots of cells isolated from the cervical LN from recipients in a. Plots were gated on live, CD3ε+ singlets. Numbers represent the frequency of cells in indicated gates. Graphs depict cumulative quantification of data pooled from three independent experiments. Symbols represent individual recipients, lines are medians. P-values were determined by unpaired Student’s t-test. (c) Representative flow cytometry plots of cells isolated from the lacrimal glands from recipients in a. Plots were gated on live, CD3ε+ singlets. Numbers represent the frequency of cells in indicated gates. Graphs depict cumulative quantification of data from one experiment (non-CD8+CD8: n = 4; CD8-only: n = 9). Symbols represent individual recipients, lines are means. P-values determined by unpaired Student’s t-test. A full color version of this figure is available at the Immunology and Cell Biology journal online.

Figure 4 CD8 and CD4 T cells each contribute to the early stages of lacrimal gland autoimmunity. Quantification of inflammation in male lacrimal glands from 11-14-week-old WT (n = 28), CD8null (n = 14) or CD4null (n = 15) NOD mice or 20-24-week-old WT (n = 7), CD8null (n = 20) or CD4null (n = 10) NOD mice. Symbols represent individual mice, lines represent medians. Significant differences between mouse groups and age groups were identified by two-way ANOVA on focus score ranks (P < 0.0001 and P < 0.01, respectively) with no significant interaction between the two (P = 0.109). Tukey’s honest significant difference post hoc test P-values are shown. *P < 0.05, ***P < 0.001 and ****P < 0.0001.

Lacrimal glands mediates epithelial cell damage, we assessed epithelial cell apoptosis by staining for activated caspase-3 in lacrimal gland sections from prior recipients of non-CD8+CD8 or CD8-only cells, or from male NOD mice that developed spontaneous dacryoadenitis. Selected recipients with focus scores similar to the median focus score of their respective group were chosen for analysis: non-CD8+CD8 (median focus score: 2.8; range: 2.2–3.2), CD8-only T cells (median focus score: 3.5; range: 1.6–9.2) and male NOD mice with spontaneous dacryoadenitis (median focus score: 8.3; range: 5.0–15.4). Additionally, recipients with no focal inflammation (i.e., focus score = 0) from each of these transfer groups were analyzed to establish baseline levels of activated caspase-3 expression. In the absence of inflammation infrequent activated caspase-3 staining was observed (0.7 activated caspase-3+ cells per mm² ± 0.5; n = 3). The total number of activated caspase-3+ cells was increased over baseline levels in non-CD8+CD8 recipient mice with inflammation (2.0 activated caspase-3+ cells per mm² ± 2.4; n = 7), in CD8-only recipients with inflammation (4.1 activated caspase-3+ cells per mm² ± 2.3; n = 7) and in mice with spontaneous inflammation (3.7 activated caspase-3+ cells per mm² ± 1.1; n = 6), although these differences did not reach statistical significance (one-way analysis of variance (ANOVA) P = 0.059). Therefore, to determine if the activated caspase-3 staining observed in the presence of inflammation could be distinguished from that observed in areas not associated with inflammation, we assessed the number of activated caspase-3+ cells based on location: within the inflammatory foci (in), outside the inflammatory foci (out) (Figure 6a) or in areas of normal tissue (normal) (Figure 6b). Within each group, more activated caspase-3+ cells were within inflammatory foci than those in the adjacent region outside the inflammation or in areas of normal tissue (Figure 6c). These data suggested an association of epithelial cell apoptosis with...
inflammatory foci, which makes biological sense. However, to mitigate potential bias associated with analysis of select areas within tissue sections, we quantified the number of activated caspase-3+ cells from whole tissue sections of glands from recipients of CD8-only cells, recipients of non-CD8+CD8 cells and male NOD mice with spontaneous disease. To confirm our findings above, we compared the total number of activated caspase-3+ cells (normalized to tissue section area) to the total number of foci (normalized to area) quantified from separate hematoxylin and eosin-stained tissue sections for each sample. As expected, these whole-slide analyses demonstrated correlation between cell death and inflammation (Figure 6d). Taken together, these findings demonstrate that epithelial cell death is associated with lacrimal gland inflammation and suggest that CD8 T cells are capable of mediating lacrimal gland epithelial cell death.

As mice in the CD8-only group included some recipients with CD4 T-cell outgrowth and also those treated with depleting antibody to prevent such outgrowth, we wanted to confirm that the number of activated caspase-3+ cells in CD8-only recipients was not dependent on an outgrowth of CD4 T cells. Thus, we assessed the correlation between the number of activated caspase-3+ cells within areas of inflammation and the percentage of CD4 T cells in the lacrimal gland 5 weeks after transfer. No correlation was observed between the number of activated caspase-3+ cells detected in inflammatory foci and the percentage of CD4 T cells within the gland (Figure 6e). Thus, these data demonstrate that epithelial cell apoptosis observed in mice with dacryoadenitis is associated with the inflammatory infiltrate in the lacrimal glands and is similar whether CD8 T cells are alone or are accompanied by other cells (e.g., CD4 T cells).
One mechanism of cell killing used by CD8 T cells is through the release of cytotoxic granules containing perforin and granzyme B.37,38 Release of these molecules is the result of degranulation, which occurs rapidly after cell activation.38 As a consequence of degranulation, CD8 T cells express the lysosomal-associated membrane glycoprotein CD107a on their surface, and this correlates with cytotoxic activity.39 Thus, measuring surface expression of CD107a identifies CD8 T cells that have undergone degranulation. To determine if CD8 T cells in the lacrimal glands displayed characteristics of cytotoxic CD8 T cells, we used flow cytometric analyses to assess the expression of CD107a and granzyme B. Assessment of lacrimal gland infiltrates isolated from male NOD mice that developed spontaneous dacryoadenitis demonstrated an increased proportion of CD8 T cells that expressed CD107a after stimulation ex vivo relative to CD8 T cells in the cervical LN (Figure 6f). Additionally, a smaller but significant increase in the percentage of CD8 T cells producing granzyme B was observed in lacrimal gland infiltrates relative to those from the cervical LN (Figure 6f). Thus, a subset of CD8 T cells within the lacrimal glands displayed a cytotoxic phenotype. Collectively, these data indicate that CD8 T cells contribute to spontaneous dacryoadenitis and induce apoptosis of lacrimal gland epithelial cells, suggesting a pathogenic role for CD8 T cells in autoimmune dacryoadenitis.

Figure 6 CD8 T cells mediate destruction of lacrimal gland epithelial cells. (a, b) Activated caspase-3 immunostaining. Representative microscopic fields of lacrimal glands with focus of inflammation (a, asterisks) or lacking inflammation (b). Arrows and inset represent activated caspase-3+ cells located within the inflammatory focus (a). Arrowheads represent activated caspase-3+ cells located outside the inflammatory cell focus (a) or in area of normal tissue devoid of inflammatory foci (b). (c) Quantification of the number of activated caspase-3+ cells in lacrimal gland sections from NOD mice that spontaneously developed lacrimal gland inflammation (n=5), or NOD-SCID mice that received CD8-only cells (n=9) or non-CD8+CD8 cells (n=4) based on location: within inflammatory foci (in), outside the inflammatory foci (out) or in areas of normal gland tissue (normal). Activated caspase-3+ cells were determined by averaging the values from two images per gland. Symbols represent individual mice, lines are means. P-values determined by two-way ANOVA demonstrates a significant difference between locations (i.e., for each group ‘in’ is greater than ‘out’ or ‘normal’) but no significant difference between groups (P=0.68) and no significant interaction between group and location (P=0.34). (d) Correlation between the total number of activated caspase-3+ cells per mm² and the number of foci per mm² in whole lacrimal gland sections from recipients of CD8-only cells (n=4), recipients of non-CD8+CD8 cells (n=4) or male NOD mice with spontaneous disease (n=5). Symbols represent individual mice. P and r-values were determined by Pearson’s correlation. (e) Correlation between the number of activated caspase-3+ cells within foci and percentage of CD4 T cells in the lacrimal glands of mice that received CD8-only T cells. Symbols represent individual mice. P and r-values were determined by unpaired Student’s t-test. A full color version of this figure is available at the Immunology and Cell Biology journal online.
DISCUSSION

CD8 T cells contribute to disease pathogenesis in a variety of autoimmune diseases. For example, CD8 T cells are required for the initiation of autoimmune diabetes and are the primary pathogenic effector cells in primary biliary cirrhosis. However, it is also well established that CD8 T cells can have a regulatory role in the context of autoimmunity. In humans with autoimmune diabetes or relapsing-remitting multiple sclerosis, a decrease in the CD8+CD28+ suppressor T-cell population has been observed. Additionally, regulatory CD8 T cells have been described in animal models of autoimmune diabetes and in experimental autoimmune encephalomyelitis, the animal model for multiple sclerosis. Thus, evidence exists for both pathogenic and regulatory roles for CD8 T cells in the context of autoimmunity.

In Sjögren syndrome, CD8 and CD4 T cells are present in lacrimal and salivary gland infiltrates from both humans and NOD mice. CD4 T cells constitute the major T-cell present in gland infiltrates, and numerous studies have investigated the contributions of these cells in Sjögren syndrome. Yet, despite the consistent presence of CD8 T cells in the lacrimal and salivary glands, it is unclear how these cells contribute to the development or progression of lacrimal gland autoimmunity. Analysis of human lacrimal gland infiltrates from patients with Sjögren syndrome found CD8 T cells clustered around apoptotic acinar epithelial cells, and increased expression of effector molecules was observed, suggesting that the cell death was mediated by CD8 T cells. A recent study found increased activated CD8 T cells in the circulation of Sjögren syndrome patients with higher disease activity. In contrast, a role for regulatory CD8 T cells has been described in a desiccating stress model resembling Sjögren syndrome. However, the role of CD8 T cells in the NOD mouse model of Sjögren syndrome has not been explored previously.

Our study demonstrates that within the lacrimal glands of male NOD mice, CD8 T cells displayed an activated phenotype, produced inflammatory cytokines upon stimulation and expressed markers indicative of cytotoxicity. Using an adoptive transfer model, our study provides evidence that CD8 T cells can independently promote dacryoadenitis and mediate epithelial cell damage. Thus, our data suggest that CD8 T cells have a pathogenic role in lacrimal gland autoimmunity.

The majority of CD8 T cells isolated from the lacrimal and salivary glands of NOD mice produced IFNγ following ex vivo stimulation, suggesting a potential mechanism by which they contribute to disease. IFNγ has multiple biological effects, including immune-cell activation, induction of chemokines and adhesion molecules on immune and nonimmune cells, and promotion of apoptosis. A pathogenic role for IFNγ has been observed in autoimmune diseases such as autoimmune diabetes, systemic lupus erythematosus and rheumatoid arthritis, and elevated levels of IFNγ were reported in Sjögren syndrome patients. In NOD mice, IFNγ was required for the development of salivary gland autoimmunity. IFNγ was shown to alter tight junction integrity and function in parotid gland epithelial cells and to induce salivary gland cell death. Further, the chemokines CXCL9 and CXCL10, which were increased in salivary gland lesions from patients with Sjögren syndrome, were induced by IFNγ in primary salivary gland epithelial cells. Thus, IFNγ likely has a pathogenic role in salivary gland disease through multiple mechanisms. Levels of CXCL9 and CXCL10 were also increased in tears from Sjögren syndrome patients, suggesting that IFNγ may have a similar role in lacrimal gland pathology. Our findings demonstrate that CD8 T cells produce IFNγ and mediate epithelial cell death in NOD mouse lacrimal glands. Thus, IFNγ likely contributes to lacrimal gland pathology through multiple mechanisms.

Because the NOD mouse model displays sex-specific differences in gland inflammation, with dacryoadenitis occurring in male mice and sialadenitis occurring in female mice, we independently studied male and female mice for the development of lacrimal and salivary gland disease, respectively. As in the male lacrimal glands, CD8 T cells were present in salivary gland infiltrates from female NOD mice, showed signs of activation and produced inflammatory cytokines. These cytokine-producing T cells were relatively more abundant within lacrimal glands, which may reflect differences in the mechanisms of disease development; however, this could also be explained by differences in the kinetics of lacrimal or salivary gland disease development. More strikingly apparent was the finding that CD8 T cells caused dacryoadenitis when transferred into male recipients, but were completely incapable of mediating sialadenitis in female recipients. Although no difference in lacrimal gland inflammation was noted on a population level between female recipients of purified CD8 T cells and non-CD8+CD8 T cells, we were surprised to find that some female recipients of purified CD8 T cells developed dacryoadenitis with focus scores greater than the median focus scores of male recipients. This is in contrast to NOD female mice or NOD-SCID female recipients of non-CD8+CD8 cells, which develop little or no lacrimal gland autoimmunity. Previously, we have shown that female NOD mice are protected from developing dacryoadenitis because of the presence of lacrimal gland-protective Tregs. One possibility here is that the transfer of purified CD8 T cells, which were largely devoid of Tregs, resulted in dacryoadenitis simply owing to the lack of lacrimal gland-protective Tregs. However, the complete absence of sialadenitis in those same recipients makes this explanation less likely because, in our hands, adoptive transfer of Treg-depleted cells (inclusive of both CD4 and CD8 T cells) results in both dacryoadenitis and sialadenitis in recipients regardless of sex (manuscript in preparation). Thus, our studies demonstrate a unique role for CD8 T cells in mediating lacrimal gland-specific inflammation in the absence of CD4 T cells.

Our studies expand previous findings that distinct mechanisms drive lacrimal and salivary gland autoimmunity. Differences in gland infiltration have been observed in NOD mice that are deficient for IFNγ or IFNγR expression. In this context, focal infiltrates in the salivary glands of NOD.IFNg−/− or NOD.IFNgR−/− mice were absent at 20 weeks of age, similar to the salivary glands from non-autoimmune, age-matched C57BL/6 mice, and in contrast to the parental NOD strain. However, leukocyte infiltrates were still observed in the lacrimal glands of NOD.IFNg−/− or NOD.IFNgR−/− mice at levels similar to that observed in NOD mice. Additionally, distinct susceptibility loci were linked to the development of dacryoadenitis and sialadenitis in the MRL/lpr mouse model of Sjögren syndrome. Thus, evidence from our study and others suggests that inflammation of the lacrimal and salivary glands can arise through distinct mechanisms.

In our adoptive transfer studies, we observed variability in disease development with some recipients failing to develop lacrimal gland inflammation. This is similar to the variability we have previously observed in this adoptive transfer model and likely reflects multiple factors. We developed this adoptive transfer model to study the early immunological events in lacrimal and salivary gland autoimmunity and believe the lack of all recipients being affected by 5–7 weeks post transfer is indicative of such an early stage in disease development. Sjögren syndrome-like disease in NOD mice is an immunologically complex disease as reflected by the variability in degree of lacrimal gland inflammation even in the spontaneous disease. In that study,

Immunology and Cell Biology
spontaneous disease in 6–12-week-old male NOD mice demonstrated variable focus scores; however, all mice in that analysis had developed some degree of dacrocyoadenitis and, in general, the focus scores in spontaneous disease were greater than those in the adoptive transfer model. It is unclear whether these differences in the transfer and spontaneous models suggest that the transfer model represents a very early stage in disease development or, rather, that other factors contributing to spontaneous disease development are not optimized in the transfer model. Regardless, it is striking that despite the variability in disease development in our adoptive transfer model, our data clearly demonstrate a pathogenic role for CD8 T cells in lacrimal gland autoimmunity including a role in mediating lacrimal gland epithelial cell death. Beyond the lacrimal glands, the ocular surface is another key site of inflammatory damage in Sjögren syndrome. Whether the pathogenic role for CD8 T cells extends similarly to the ocular surface in NOD mice was not evaluated in our studies. Interestingly, studies in a desiccating stress model demonstrated a regulatory role for CD8 T cells at the ocular surface, so further studies into the role of CD8 T cells in ocular surface inflammation in NOD mice are warranted.

In addition to a pathogenic role for CD8 T cells, our studies here also further support the key role of CD4 T cells in lacrimal gland autoimmunity as evidenced by the outgrowth of CD4 T cells and their enrichment within lacrimal glands in our CD8-only transfer studies. Because we focused on CD8 T cells in these studies, we did not evaluate for phenotypic differences in the outgrowing CD4 T-cell population compared with CD4 T cells in non-CD8+CD8 transfers, non-CD8 transfers or spontaneous disease. Further study of these CD4 T-cell populations may provide insight into the specific mechanisms of pathogenic CD4 T-cell involvement in lacrimal gland autoimmunity. Regardless, our data demonstrate that even when the outgrowth of CD4 T cells is prevented, CD8 T cells are capable of mediating lacrimal gland autoimmunity.

Understanding the initiation of a complex autoimmune disease such as Sjögren syndrome requires knowledge of all of the cellular players involved in the immune response. Our work here describes a pathogenic role for CD8 T cells in the early stages of dacrocyoadenitis. Further insight into the mechanisms that are required for CD8 T cells to overcome immune tolerance will help in understanding the events that lead to the development of organ-specific autoimmunity.

**METHODS**

**Mice**

Male and female NOD/ShiLtJ (NOD), NOD.CB17-PrkaCAT½f (NOD-SCID), NOD.129S2(B6)-Cdx2fllox/flox(Dys) (CDH4)(fl)/ and Balb/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Male and female NOD.129S2(B6)-Cdx2fllox/flox(Dys) (CDH4)(fl) mice were kindly provided by David Serreze (The Jackson Laboratory). NOD mice expressing the bicistronic fluorescent protein knock-in C57BL/6 J mice56 for 17 weeks old. The exact sample size (n) and biological and experimental replicates are indicated in each figure legend. Samples sizes were determined based on previous transfer studies and typically included 10–15 mice per group pooled from multiple experiments. All mice were monitored for the presence of glucosuria using Diastix urine dipsticks (Bayer Diagnostics, Whippany, NJ, USA). A positive test was indicative of autoimmune diabetes development. Mice were maintained and used in accordance with the University of Iowa Institutional Animal Care and Use Committee Guidelines.

**Histological characterization of inflammation in lacrimal and salivary glands**

Exorbital lacrimal and submandibular salivary glands were harvested and fixed in buffered formalin, processed, embedded in paraffin and sectioned. Five micrometer sections of paired glands were stained with hematoxylin and eosin and analyzed by standard light microscopy. Inflammation was quantified using standard focus scoring. Focus scores (number of inflammatory foci per 4 mm²) were calculated by a blinded observer by counting the total number of foci (composed of ≥ 50 mononuclear cells) by standard light microscopy using a x10 objective, scanning slides to obtain digital images using PathScan Enabler IV (Meyer Instruments, Houston, TX, USA), and measuring surface area of sections using the ImageJ software (US National Institutes of Health, Bethesda, MD, USA). Samples with diffuse inflammation resulting in coalescence of individual foci were assigned focus score values greater than the highest calculable value for that set of comparisons. Representative images were captured on a Leitz DM-2B research microscope with a Leica DCF700T digital camera using the Leica Application Suite X software (Leica Microsystems, Wetzlar, Germany).

**Lymphocyte isolation**

Cervical LN or spleens were dissociated with the end of a 3 ml syringe plunger through 70 μm nylon mesh in RPMI (Life Technologies, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 50 μg β-mercaptoethanol (complete RPMI). Cells were treated with ACK lysis buffer (Lonza, Mapleton, IL, USA) to remove red blood cells. To isolate cells from the lacrimal or salivary glands, glands were incubated in Collagenase type IV (Life Technologies, Waltham, MA, USA) at 37 °C with shaking for 1 or 1.5 h, respectively. Glands were then dissociated with the end of a 3 ml syringe plunger through 40 μm nylon mesh and red blood cells lysed as above to obtain single-cell suspensions. Peripheral blood samples were collected through the retro-orbital plexus and diluted in phosphate-buffered saline containing 20 U ml⁻¹ heparin sodium (Fisher Scientific, Hampton, NH, USA). Single-cell suspensions were obtained using Histopaque-1083 (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer’s instructions.

**Flow cytometry and FACS**

Cells from cervical LN, lacrimal or salivary glands, or peripheral blood were analyzed by flow cytometry using a BD LSR II or BD Acurri C6 (BD Biosciences, San Jose, CA, USA) for acquisition and the FlowJo software (Treestar Inc., Ashland, OR, USA) for analysis. All samples were gated initially on forward and side scatter parameters to establish the lymphocyte gate and then on forward scatter-area by forward scatter-width to establish singlet gates. Specific additional gating was noted in figure legends. For live/dead discrimination, cells were stained with a fixable viability dye from eBioscience (San Diego, CA, USA). Intracellular staining was performed using the Foxp3/transcription factor staining buffer set according to the manufacturer’s instructions (eBioscience). For CD8-based FACS, cells were labeled with a fluorescein-conjugated anti-CD8α monoclonal antibody and sorted into CD8⁺ and CD8⁻ populations using a FACS Aria. For analyses of peripheral blood cells from mice treated with anti-CD4 antibody (GK1.5), staining was performed using different anti-CD4 antibody clone (RM4-5). For cytokine analyses, cells were stimulated for 4 h at 37 °C with Leukocyte Activation Cocktail (with BD GolgiPlug) plus monensin (BD Biosciences) in complete RPMI followed by surface and intracellular staining. For CD107a analysis, cells were stimulated as for cytokine analysis, with the anti-CD107a antibody included during cell stimulation. Antibodies were purchased from BD Biosciences, Biologend (San Diego, CA, USA) or eBioscience. Antibody clones used were as follows: CD3e (145-2c11), CD4 (GK1.5 or RM4-5), CD8α (53-6.7), CD11a (2D7), CD69 (H1.2F3), CD107a (1D4B), IFNγ (XMG1.2), Foxp3 (FJK-16sc), Ki67 (SolA15) and tumor necrosis factor α (MP6 XT22).

**Anti-human granuzyme B (GB12) was obtained from Life Technologies.**

**Transfer model of Sjögren syndrome**

Donor cells were isolated from cervical LN (mandibular, accessory mandibular and superficial parotid), pooled from several sex-matched NOD mice and
adoptively transferred intravenously to sex-matched NOD-SCID recipients. Recipients of different donor cell groups were randomly assigned with each cage housing recipients from multiple groups to avoid a cage effect. Transferred cells included 1.5–2 × 10^6 purified CD8 T cells (CD8-only), 5 × 10^6 CD8-depleted cells (non-CD8) or a combination of 1.5–2 × 10^6 CD8-only and 5 × 10^6 non-CD8 (non-CD8+CD8). After 5–7 weeks, organs were harvested for histology and/or flow cytometric analysis. For anti-CD4 treatment, mice received weekly intraperitoneal injections of 100 μg of anti-CD4 antibody (GK1.5) or isotype control antibody (rat IgG2b anti-KLH) (BioXcell, Lebanon, NH, USA) beginning at the time donor cells were transferred. All donors and recipients tested negative for glucosuria at the time of killing for tissue harvesting.

Activated caspase-3 staining

Activated caspase-3 immunostaining, which is a recommended and validated technique for detecting and quantifying apoptotic cells in tissue sections, was performed similar to previous methods. Briefly, paraffin-embedded tissues were sectioned (~4 μm) onto slides, and hydrated through a series of xylene and alcohol baths. Epitope unmasking was performed using heat-induced antigen retrieval (citrate buffer (pH 6.0), 125 °C × 25 min). Primary antibody was a rabbit polyclonal antibody (1:100, cat. no. 9661; Cell Signaling Technology, Danvers, MA, USA). 3,3’-Diaminobenzidine was the chromogen, and hematoxylin was the counterstain. Activated caspase-3 staining was quantified using a postexamination masking technique to mitigate bias. Briefly, high-resolution images at ≥200 magnification were collected using a BX51 microscope, DP53 camera and CellSens Software (Olympus Corporation, Center Valley, PA, USA). A deconvolution extension was used to remove the effects of overlapping pixels and a median filter was applied to remove any remaining image noise. Activated caspase-3 staining was quantified using a post-examination masking technique to mitigate bias. For each gland, the average number of activated caspase-3+ cells was obtained from two images. For whole-section activated caspase-3+ cell quantitation, the number of activated caspase-3+ cells within whole tissue sections from paired glans was obtained. For each sample, the number of activated caspase-3+ cells was enumerated and normalized to total tissue section area to obtain the number of activated caspase-3+ cells per mm². These data were then used in correlation analyses to determine the degree of correlation to the number of foci per mm² obtained from hematoxylin- and eosin-stained sections from the same tissue samples. All analyses were carried out in a masked manner.

Statistical analysis

Statistical analyses were performed with Prism software version 6.0 (GraphPad, San Diego, CA, USA) or R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). Mann–Whitney U-test was used for two-group comparisons of non-normally distributed data (focus scores). Unpaired Student’s t-test was used for two-group comparisons of data that approximated normal distribution (flow cytometry data). Welch’s correction was applied to comparisons with significant differences in variance. All two-group comparisons were two-tailed. Two-way ANOVA was used for two-dimensional analyses. For two-dimensional analyses of non-normally distributed data, the observed data values were converted to ranks, two-way ANOVA was performed on those ranks, and Tukey’s honest significant difference post hoc test was used to control the α-level for multiple comparisons. Visual inspection of the residuals yielded no evidence of any model assumptions being violated. Pearson’s correlation coefficient was calculated for correlation analysis of normally distributed data. Spearman’s correlation coefficient was calculated for correlation analysis of non-normally distributed data. P-values < 0.05 were considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Stanley Perlman for critical reading of the manuscript and helpful suggestions, the Karandikar lab for insightful discussions, Dr David Serreze and Harold Chapman for assistance with initial CDMnull mouse studies, Dr Vijay Kuchroo for mice, and Dr Jacob Oleson and Uchechuku Nwoke for assistance with statistical analyses. This work was supported by the National Institutes of Health (K08 EY022344 to SML) and start-up funds from the Stead Family Department of Pediatrics (to SML). The data presented herein were obtained at the Flow Cytometry Facility, which is a Carver College of Medicine/Holden Comprehensive Cancer Center core research facility at the University of Iowa. The Facility is funded through user fees and the generous financial support of the Carver College of Medicine, Holden Comprehensive Cancer Center and Iowa City Veteran’s Administration Medical Center. Research reported in this publication was supported by the National Center for Research Resources of the National Institutes of Health under Award Number 1 S10 OD016199-01A1.

1. Mavragani CP, Moutsopoulos HM. Sjögren’s syndrome. Annu Rev Pathol 2014; 9: 137–61.
2. Maslinska M, Przygoda M, Kwiatkowska B, Sikorska-Suđek K. Sjögren’s syndrome: still not fully understood disease. Rheumatol Int 2015; 35: 233–241.
3. Theander E, Jonsson R, Stjörom B, Brokstad K, Olsson P, Henriksson G. Prediction of Sjögren’s syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling. Arthritis Rheumatol 2015; 67: 2427–2436.
4. Delaleu N, Nguyen CQ, Peck AB, Jonsson R. Sjögren’s syndrome: studying the disease in mice. Arthritis Res Ther 2011; 13: 217.
5. Lee BH, Gauna AE, Pauley KM, Park YJ, Cha S. Animal models in autoimmune diseases: lessons learned from mouse models for Sjogren’s syndrome. Clin Rev Allergy Immunol 2012; 42: 35–44.
6. Lieberman SM, Kreiger PA, Koretsky GA. Reversible lacrimal gland-protective regulatory T-cell dysfunction underlies male-specific autoimmune dacryoadenitis in the non-obese diabetic mouse model of Sjögren’s syndrome. Immunology 2015; 145: 232–241.
7. Mikulowska-Mennis A, Xu B, Berberian JM, Michie SA. Lymphocyte migration to inflamed lacrimal glands is mediated by vascular cell adhesion molecule-1 (VCAM-1) integrin, peripheral node addressin/slectin, and lymphocyte function-associated antigen-1 adhesion pathways. Am J Pathol 2001; 159: 671–681.
8. Toda I, Sullivan BD, Rocha EM, Da Silveira LA, Wickham LA, Sullivan DA. Impact of gender on exocrine gland inflammation in mouse models of Sjögren’s syndrome. Exp Eye Res 1999; 69: 355–366.
9. Hunger RE, Carnaud C, Vogt I, Mueller C. Male gonadal environment paradoxically promotes dacryoadenitis in nonobese diabetic mice. J Clin Invest 1998; 101: 1300–1309.
10. Takahashi M, Ishimaru N, Yanagi K, Haney N, Saito I, Hayashi Y. High incidence of autoimmune dacryoadenitis in male non-obese diabetic (NOD) mice depending on sex steroid. Clin Exp Immunol 1997; 109: 559–561.
11. Fujihara T, Fujita H, Tsubota K, Saito K, Tsuzaki K, Abe T et al. Preferential localization of CD8+ alpha E beta 7+ T cells around acinar epithelial cells with apoptosis in patients with Sjögren’s syndrome. J Immunol 1999; 163: 2226–2235.
12. Robinson CP, Cornelius J, Bourous DE, Yamamoto H, Humphreys-Beher MG, Peck AB. Characterization of the changing lymphocyte populations and cytokine expression in the exocrine tissues of autoimmune NOD mice. Autoimmunity 1998; 27: 29–44.
13. Matsumoto I, Tsubota K, Satake Y, Kita Y, Matsuruma R, Murata H et al. Common T cell receptor clonotype in lacrimal glands and labial salivary glands from patients with Sjögren’s syndrome. J Clin Invest 1996; 97: 1969–1977.
14. Christodouli MI, Kapsogeorgos EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren’s syndrome. J Autoimmun 2010; 34: 400–407.
15. Sekai A, Sugawara Y, Kunishi T, Sasano T, Sugawara S. Identification of IL-18 and TNFα cells in salivary glands of patients with Sjogren’s syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18. J Immunol 2008; 181: 2988–2996.
16. Mingione S, Boudaud S, Haskell S, Reynolds TL, Nocturne G, Norton E et al. Cytometry by time-of-flight immunophenotyping identifies a blood Sjogren’s signature correlate with disease activity and glandular inflammation. J Allergy Clin Immunol 2016; 137: 1809–1821.
17. Yang GX, Wu Y, Tsukamoto H, Leung PS, Lian ZX, Rainbow DB et al. CD8 T cells mediate direct biliary ductule damage in nonobese diabetic autoimmune biliary disease. J Immunol 2011; 186: 1259–1267.
18. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD NON-Thy-1a donors. Diabetes 1993; 42: 44–55.
19. Di Lorenzo TP, Graser RT, Oso T, Christianson GJ, Chapman HD, Rooenpen DC et al. Major histocompatibility complex class I-restricted T cells are required for all but the early stages of diabetes development in nonobese diabetic mice and use a prevalent T cell receptor alpha chain gene rearrangement. Proc Natl Acad Sci USA 1998; 95: 12538–12543.
CB6 T cells promote lacrimal gland autoimmune

Immunology and Cell Biology

694

20 Hayward SL, Bautista-Lopez N, Suzuki K, Atrasheh A, Dicke P, Elliott JF. CD4 T cells play major effector role and CD8 T cells initiating role in spontaneous autoimmune myocarditis of HLA-DQB1 transgenic IAb knockout nonobese diabetic mice. J Immunol 2006; 176: 7715–7725.

21 Tsai S, Clemente-Casares X, Santamaría P. CD4<sup>+</sup> Tregs in autoimmunity: learning "self" control from experience. Cell Mol Life Sci 2011; 68: 3781–3795.

22 Mikulová Z, Praksova P, Stourac P, Bednarik J, Svitálová L, Pacasová R et al. Numerical defects in CD8<sup>+</sup>CD28<sup>+</sup> T-suppressor lymphocyte population in patients with type 1 diabetes mellitus and multiple sclerosis. Cell Immunol 2010; 262: 75–79.

23 Najdian N, Chitnis T, Salama AD, Zhu B, Beno C, Yuan X et al. Regulatory functions of CD8<sup>+</sup>CD28<sup>+</sup> T cells in an autoimmune disease model. J Clin Invest 2003; 112: 1037–1048.

24 Abdul-Majid KB, Weber J, Stadelmann C, Steffert A, Lasmann H, Olsson T et al. Comparing the pathogenesis of experimental autoimmune encephalomyelitis in CD4<sup>+</sup> and CD8<sup>+</sup> DBA/1 mice defines qualitative roles of different T cell subsets. J Neuroimmunol 2003; 141: 10–19.

25 Tsai S, Shamiel A, Yamanoichi J, Clemente-Casares X, Wang J, Serra P et al. Reversal of autoimmunity by boosting memory-like autoregulatory T cells. Immunol 2010; 32: 568–580.

26 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

27 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

28 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

29 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

30 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

31 Van Severen GA, Shimizu Y, Horgan KJ, Shaw S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J Immunol 1990; 144: 4579–4586.

32 Bachmann MF, McHaii-Faienza K, Schmits R, Bouchard D, Beach J, Speiser DE et al. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. Immunology 1997; 7: 549–557.

33 Rai D, Pham NL, Harty JT, Badovinac VP. Tracking the total CD8 T cell response to CD4<sup>+</sup> cells regulate the T helper-17 response in an experimental murine model of Ectopic lymphoid structure and function in the rat parotid gland Par-C10 cell line. Am J Physiol Cell Physiol 2008; 295: C1191–C1201.

34 Rai D, Pham NL, Harty JT, Badovinac VP. Tracking the total CD8 T cell response to CD4<sup>+</sup> cells regulate the T helper-17 response in an experimental murine model of Ectopic lymphoid structure and function in the rat parotid gland Par-C10 cell line. Am J Physiol Cell Physiol 2008; 295: C1191–C1201.

35 Matsumura R, Unminya K, Goto T, Nakazawa T, Ochiai K, Kagami M et al. Interferon-gamma and tumor necrosis factor-alpha induce alpha factor and anti-Fas mediated apoptosis in a salivary ductal cell line. Cell Immunol 2000; 1(5): 38–48.

36 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

37 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

38 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

39 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

40 Taneja V, Taneja N, Paisansinsup T, Behrens M, Grif

41 Zhang X, Schaumburg CS, Coursey TG, Siemasko KF, Volpe EA, Gandhi NB et al. Intracellular cytokine optimization and standard operating procedure. Nat Protoc 2006; 1: 1507–1516.

42 Van den Broeck W, Derore A, Simoons P. Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNcr mice. J Immunol Methods 2006; 312: 12–19.

43 Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS, Blomme EA. Comparison of immunohistochemical staining for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC<sub>3</sub> subcutaneous xenografts. J Pathol 2003; 199: 221–228.

44 Price LC, Shao D, Meng C, Peros F, Garfield BE, Zhu J et al. Dexamethasone induces apoptosis in pulmonary arterial smooth muscle cells. Respir Res 2015; 16: 114.

45 Finkin S, Yuan D, Stein I, Taniguchi K, Weber A, Ungler K et al. Ectopic lymphoid structures function as micromilieu for tumor progenitor cells in hepatic fibrosis. Nat Immunol 2015; 16: 1235–1244.

46 Chen YC, Kuo HY, Borrischein U, Takahashi H, Chen SY, Lu KM et al. Foxp2 controls synaptic wiring of corticostriatal circuits and vocal communication by opposing MeT<sub>2</sub>. Nat Neurosci 2016; 19: 1513–1522.

47 Dai Y, Jia P, Fang Y, Liu H, Jiao X, Jiang H et al. miR-146A is essential for lymphoproliferative (LPS)-induced cross-tolerance against kidney ischemia/reperfusion injury in mice. Sci Rep 2016; 6: 27091.

48 Hunninghake GW, Doerschuk KC, Nymon AB, Schmidt GA, Meyerholz DK, Ashare A. Insulin-like growth factor-1 levels contribute to the development of bacterial translocation in sepsis. Am J Respir Crit Care Med 2010; 182: 517–525.

49 Gibson-Corley KN, Olliver AK, Meyerholz DK. Principles for valid histopathologic scoring of CD8 T cells promote lacrimal gland autoimmune

The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/ib)