Th1 Versus Th2 Immune Responses in Autoimmune Lacrimal Gland Disease in MRL/Mp Mice

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PURPOSE. In MRL/Mp-lpr/lpr (MRL/lpr) and MRL/Mp+/+ (MRL+/+) mice, a T-cell–driven lacrimal gland inflammation spontaneously develops that is a model for Sjögren’s syndrome. The lacrimal gland lesions in these mice were evaluated by immunohistochemistry for the relative contributions of T-helper (Th)1 versus Th2 immune responses.

METHODS. Frozen sections of lacrimal glands from MRL/lpr and MRL+/+ mice ages 1 through 5 months were stained with monoclonal antibodies to the cytokines interferon (IFN)-γ and interleukin (IL)-4 and to the cell surface costimulatory molecules B7-1 and B7-2, which are associated with Th1 and Th2 responses, respectively.

RESULTS. The median proportion of cells staining for IL-4 ranged from 30% to 67% over time for MRL/lpr mice and from 30% to 55% for MRL+/+ mice. The median proportion of cells staining for IFN-γ ranged from 1% to 4% for MRL/lpr mice and from 0% to 3% for MRL+/+ mice. The proportion of cells staining positively for IL-4 was significantly greater than for IFN-γ in both MRL/lpr (mean difference, 33%; P = 0.0001) and MRL+/+ (mean difference, 42%; P = 0.0002). The median proportion of cells staining positively for B7-2 ranged from 20% to 38% for MRL/lpr mice and from 16% to 34% for MRL+/+ mice. The median proportion of cells staining for B7-1 ranged from 2% to 10% for MRL/lpr mice and from 2% to 5% for MRL+/+ mice. The proportion of cells staining positively for B7-2 was significantly greater than for B7-1 for both MRL/lpr mice (mean difference, 15%; P = 0.001) and for MRL+/+ mice (mean difference, 19%; P = 0.006).

CONCLUSIONS. On the basis of immunohistochemistry for cytokines and costimulatory molecules, inflammatory lacrimal gland lesions in MRL/lpr and MRL+/+ mice appear to be a largely Th2 phenomenon. (Invest Ophthalmol Vis Sci. 2000;41:826–831)

Autoimmune disease, including lacrimal gland inflammation, develops spontaneously in MRL/Mp mice, providing a model for the human disorder Sjögren’s syndrome.1–6 There are two congenic substrains of MRL/Mp mice that differ only by a single autosomal recessive mutation, the lpr gene.1,2 The lpr mutation results in an altered Fas protein and defective lymphocytic apoptosis, and appears to cause defective clonal deletion of autoreactive T cells in peripheral lymphoid organs and defective elimination of activated T cells after response to antigen.7–9 Results of this defective apoptosis include accelerated autoimmune disease in MRL/Mp-lpr/lpr (MRL/lpr) when compared with MRL/Mp+/+ (MRL+/+) mice and a massive accumulation of Thy 1.2+, CD4+, CD8+, TCR-α/β+ “double-negative” T cells in the lymph nodes.1,2,10–12 Although an accelerated autoimmune disease develops in MRL/lpr mice, inflammatory lacrimal gland lesions, which are composed largely of T cells (approximately 80%), the majority (approximately 65%–74%) of which are CD4+ T cells, develop in both substrains.4–6 Although inflammatory lacrimal gland lesions develop in both MRL/lpr and MRL+/+ mice, there are differences between the two substrains. Lacrimal gland disease develops earlier in MRL/lpr mice than in MRL+/+ mice, and at comparable ages MRL/lpr mice have more severe disease. Furthermore, a late accumulation of B cells develops in the lacrimal gland lesions in MRL/lpr mice that is not seen in MRL/lpr mice and suggests that MRL+/+ and MRL/lpr mice could have different immunologic mechanisms of autoimmune lacrimal gland disease.

CD4+ helper T (Th) cells differentiate into two subpopulations, Th1 and Th2, with different effector mechanisms.13–19 Th1 cells produce interferon (IFN)-γ and tumor necrosis factor (TNF) and are primarily responsible for cell-mediated immune responses, such as delayed-type hypersensitivity. Th2 cells produce interleukin (IL)-4, IL-5, and IL-10 and provide help to B cells in antibody production in humoral immune responses.15,18 Factors involved in directing the immune response toward Th1 or Th2 include specific cytokines; IL-12 and IFN-γ induce a Th1 response, whereas IL-4 induces a Th2 response, and IL-10 inhibits a Th1 response. Certain antigens are more likely to induce a predominant subset as well.19,20 B7 is a costimulatory molecule expressed on antigen-presenting cells and is required for the effective stimulation of T cells to respond to antigen presentation; it exists as two subtypes, B7-1 and B7-2, which appear to stimulate Th1 responses and Th2 responses, respectively.21,22

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We report the results of immunohistochemistry to evaluate the relative roles of Th1 versus Th2 responses in the inflammatory lacrimal gland lesions of both substrains of MRL/Mp mice.

**MATERIALS AND METHODS**

**Mice**

MRL/Mp mice, of both substrains, and control BALB/c mice were obtained from the Jackson Laboratories (Bar Harbor, ME) at 1 month of age and kept under standard conditions in the animal facilities of the Woods Research Building of the Johns Hopkins Hospital until killed. Groups of five mice of each strain were anesthetized and killed by exsanguination at ages 1, 2, 3, 4, and 5 months. At the time of death, lacrimal glands were removed, embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN), frozen in liquid nitrogen, sectioned at 8 μm on a cryostat, and stained as described later. These experiments conformed to the ARVO Statement for the Care and Use Committee.

**Immunocytochemistry**

Staining of frozen sections of the lacrimal glands was performed using a panel of monoclonal antibodies and the avidin-biotin peroxidase complex (ABC) technique. Frozen sections were fixed in chilled (4°C) acetone, air dried, rehydrated in phosphate-buffered saline (PBS), and incubated with the appropriate blocking agent (Vector, Burlingame, CA) for 15 minutes. The primary antibody was applied, and the slides were incubated for 60 minutes. A second blocking step was then performed. The slides were washed in PBS, incubated with a biotinylated secondary antibody for 30 minutes, rinsed in PBS, incubated with the ABC agent for 45 minutes, washed again in PBS, developed with 3% hydrogen peroxide and 3-amin-9-ethyl-carbazole (Vector), as for single staining, and then a hamster anti-mouse CD3 monoclonal antibody (gift of James P. Allison) or the rat anti-mouse CD4 antibody (PharMingen), and the ABC technique using 3-amino-9-ethyl-carbazole (Vector), as for single staining. Double staining for IL-4 and either CD3 or CD4 was performed using a rat anti-mouse IL-4 antibody (PharMingen), and the ABC technique using 3-amino-9-ethyl-carbazole (Vector), as for single staining, and then a hamster anti-mouse CD3 monoclonal antibody (gift of James P. Allison) or the rat anti-mouse CD4 monoclonal antibody, anti-L3T4 (Becton Dickinson, Bedford, MA), and the ABC technique, using glucose oxidase (Vector).

**Statistics**

The comparison of the proportion of cells staining positively for IL-4 and IFN-γ and for B7-2 and B7-1 was performed using a sign test, a nonparametric paired analysis. The evaluation of trends over time for cytokines or cell surface markers within a substrain was performed using linear regression; the results are expressed as β, the slope of the regression line. Comparison between substrains was performed using an analysis of covariance.

**RESULTS**

Results of the immunocytochemistry from mice aged 2 to 5 months are outlined in Table 1. Both MRL/lpr (Fig. 1) and MRL/+ mice (Fig. 2) showed a markedly increased proportion of cells staining for IL-4 and very few cells staining for IFN-γ. Double staining performed on three 3- to 4-month-old MRL/lpr mice and on three 3- to 4-month-old MRL/+ mice confirmed that nearly all the IL-4–stained cells were CD3+CD4+ T cells. The median proportion of cells that stained positively for IL-4 ranged from 30% to 67% for MRL/lpr mice, and 30% to 55% for MRL/+ mice, whereas the median proportion of cells that

**TABLE 1. Immunocytochemistry of Inflammatory Lacrimal Gland Lesions in MRL/Mp Mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (mo)</th>
<th>IL-4</th>
<th>IFN-γ</th>
<th>B7-1</th>
<th>B7-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRL/lpr</td>
<td>2</td>
<td>30 (10–42)</td>
<td>5 (2–13)</td>
<td>10 (6–30)</td>
<td>26 (22–29)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>67 (50–69)</td>
<td>1 (0–8)</td>
<td>10 (5–15)</td>
<td>20 (19–28)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30 (2–35)</td>
<td>3 (2–7)</td>
<td>2 (5–17)</td>
<td>38 (21–40)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>40 (3–60)</td>
<td>3 (0–8)</td>
<td>6 (4–10)</td>
<td>20 (12–22)</td>
</tr>
<tr>
<td>MRL/+</td>
<td>2</td>
<td>30 (18–45)</td>
<td>1 (1)</td>
<td>5 (3–23)</td>
<td>16 (15–26)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>46 (35–65)</td>
<td>0 (0–9)</td>
<td>2 (0–4)</td>
<td>22 (0–23)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>40 (22–53)</td>
<td>1 (0–5)</td>
<td>2 (1–19)</td>
<td>34 (33–40)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>55 (40–60)</td>
<td>3 (2–4)</td>
<td>4 (2–15)</td>
<td>28 (22–44)</td>
</tr>
</tbody>
</table>

Median % cells staining positively; range in parentheses. For MRL/lpr mice five sections were available for all analyses. For MRL/+ mice, limited tissue with lesions led to four sections being available for some analyses, and in one case (B7-1 in 2-month-old mice) only three sections were available.
stained positively for IFN-γ ranged from 1% to 5% for MRL/lpr mice, and from 1% to 3% for MRL/+ mice. The proportion of cells staining positively for IL-4 was significantly greater than the proportion staining positively for IFN-γ (mean difference, 33%; \( P = 0.001 \)) and for MRL/+ mice (mean difference, 42%; \( P = 0.002 \)). Immunocytochemistry for B7-1 and B7-2 revealed that a greater percentage of cells stained for B7-2 than for B7-1 for both MRL/lpr (Fig. 3) and MRL/+ mice (Fig. 4). The median proportion of cells staining positively for B7-2 ranged from 20% to 58% for MRL/lpr mice and from 16% to 34% for MRL/+ mice, whereas the median proportion of cells staining positively for B7-1 ranged from 2% to 10% for MRL/lpr mice and from 2% to 5% for MRL/+ mice. There was a significantly greater proportion of cells staining positively for B7-2 in both MRL/lpr mice (mean difference, 15%; \( P = 0.001 \)), and in MRL/+ mice (mean difference, 19%; \( P = 0.006 \)). One-month-old MRL/lpr mice typically had lesions too small to be analyzed, although in two mice staining for IL-4 and B7-2 was evident, whereas staining for IFN-γ and B7-1 was not seen. One-month-old MRL/+ mice had no lesions.

**Temporal Trends**

There were no significant trends over time in the proportion of cells staining positively for IL-4 in MRL/lpr mice (\( \beta = -1.2; P = 0.75 \)) or MRL/+ mice (\( \beta = 4.8; P = 0.14 \)) or for IFN-γ in MRL/lpr mice (\( \beta = -0.7; P = 0.29 \)) or MRL/+ mice (\( \beta = 0.48; P = 0.50 \)). For B7-2 there was no significant trend over time for MRL/lpr mice (\( \beta = -1.96; P = 0.17 \)), but there was a signifi-

**FIGURE 1.** Inflammatory lacrimal gland lesion from a 3-month-old MRL/lpr mouse stained with monoclonal antibody to (A) IL-4 and (B) IFN-γ. A large number of cells staining for IL-4 was present within the lesion, whereas cells staining for IFN-γ were only sparsely present. Original magnification, ×200.

**FIGURE 2.** Inflammatory lacrimal gland lesion from a 5-month-old MRL/+ mouse stained with monoclonal antibody to (A) IL-4 and (B) IFN-γ. Many cells staining for IL-4 were present within the lesion and surround residual glandular acini, whereas cells staining for IFN-γ were only sparsely present. Original magnification, ×200.
cant increase over time in MRL/+ mice ($\beta = 5.45; P = 0.015$). For B7-1 there was no significant trend over time for MRL/+ mice ($\beta = -0.41; P = 0.80$), but there was a suggestion of a decline over time for MRL/lpr mice ($\beta = -2.14; P = 0.08$), which did not reach the conventional level of significance of $P = 0.05$.

**Comparison of MRL/lpr and MRL/+ Mice**

There was no significant difference between the two sub-strains for IL-4 staining. For IFN-$\gamma$, there was a suggestion that MRL/lpr mice had a greater proportion of cells staining positively than did MRL/+ mice ($P = 0.078$), and for B7-1, there was a suggestion that MRL/lpr mice had a greater percentage of cells staining positively than did MRL/+ mice ($P = 0.064$). However, neither of these differences was at the conventional level of significance of $P = 0.05$. Because of the significant sub-strain by time interaction (different slopes on the regression lines) for B7-2 staining, the two strains could not be compared directly for the proportion of cells staining positively for B7-2 overall.

Age-matched control BALB/c mice showed no lacrimal gland inflammation at any of the ages studied.

**DISCUSSION**

Previous work has demonstrated that the inflammatory lacrimal gland lesions of MRL/lpr and MRL/+ mice are composed largely of CD4$^+$ T cells (approximately one half to two thirds) with lesser numbers of CD8$^+$ T cells, macrophages, and B cells.$^4$ The results are similar to those seen in minor salivary gland biopsies from patients with Sjögren's syndrome, where more than 75% of the infiltrating lymphocytes are T cells and 50% to 75% are CD4$^+$ T cells.$^{29,30}$ The results from the
current experiments suggest that the lacrimal gland lesions in both MRL/lpr and MRL/+ are largely Th2. From the earliest inception of the inflammatory lacrimal gland lesions in both MRL/lpr and MRL/+ substrains, the principal cytokine present within cells comprising the inflammatory lesion is IL-4, with little IFN-γ. Although IFN-γ can be detected within a few isolated cells in the inflammatory lacrimal gland lesions, IL-4 predominates, suggesting that the lesions are largely Th2. Our results show that B7-2 is the major costimulatory molecule seen in the lacrimal gland lesions of both MRL/lpr and MRL/+ mice. It has been reported by Kuchroo et al. that B7-1 is the costimulatory molecule for Th1 cells and that B7-2 is the costimulatory molecule for Th2 cells. The finding of a predominance of B7-2 within the lacrimal gland lesions of MRL/lpr and MRL/+ mice further suggests a Th2 response at this site.

Because of the known late accumulation of B cells in the lacrimal gland lesions of MRL/+ mice, we had initially hypothesized that the lesions in MRL/+ mice may be Th2. The predominance of IL-4 over IFN-γ and B7-2 over B7-1 and the increase in B7-2 with time are all consistent with Th2 response in this strain. However, Murray et al. and Takahashi et al. have reported that the autoimmune mechanism in MRL/lpr mice spleens and lymph nodes is Th1. As such, it was possible that the lacrimal gland lesions in MRL/lpr mice might be predominately Th1, predominately Th2, or mixed. Because of the accelerated lacrimal gland disease seen in MRL/lpr mice, we initially predicted that a mixed population would be present in MRL/lpr mice with an increasing Th1 component over time. However, our results demonstrate a predominant Th2 response in MRL/lpr mice. Although the numbers of positively staining cells are small, the suggestion of an increase in staining for IFN-γ and B7-2 in the lacrimal gland lesions of MRL/lpr mice compared with MRL/+ mice and the earlier onset of disease in these mice are both consistent with a mild influence on the lpr gene on the lacrimal gland lesions in MRL/lpr mice. However, the predominant character of the lacrimal gland lesions in MRL/lpr mice remains that of the background MRL/Mp strain (i.e., a Th2 response).

Our results demonstrating that the lacrimal gland lesions are largely Th2 are consistent with the fact that the lacrimal gland disease is intrinsic to the MRL/Mp mouse and are present in both substrains. The lpr gene accelerates the development of the autoimmune disease present in MRL/+ mice but is not required for the lacrimal gland disease. Luzina et al. have recently demonstrated that the infiltrating lymphocytes in the vasculitic lesions of Palmerston North mice, another autoimmune strain of mice, are largely Th2, also suggesting that Th2 responses can be responsible for autoimmune end-organ disease.

Evaluations of minor salivary biopsy specimens from patients with Sjögren's syndrome have given inconsistent results. Fox et al. reported that the lymphocytes from minor salivary gland biopsy specimens transcribed mRNA for IL-2, IFN-γ, and IL-10, but little IL-4. The coproduction of IFN-γ and IL-10 was considered peculiar, because IFN-γ generally is associated with Th1 responses and IL-10 with Th2 responses. Conversely, Ohyama et al. detected IL-2 and IFN-γ mRNA consistently in minor salivary gland biopsy specimens but also detected IL-4 mRNA in specimens with an accumulation of B cells. These results suggest a less clear-cut distinction between Th1 and Th2 responses in minor salivary gland biopsy specimens of patients with Sjögren's syndrome, at least when cytokines are evaluated by reverse transcription-polymerase chain reaction for cytokine mRNA transcription.

The autoimmune lymphoproliferative syndrome is a recently described human disorder similar to that seen in MRL/lpr mice. Patients with this disorder have an inherited defect of apoptosis, generally caused by a defective Fas protein, lymphoproliferation, excess numbers of CD3+ CD4− CD8− double-negative lymphocytes, autoimmune disease, and autoantibodies. Evaluations of Th1 versus Th2 responses in these patients show a prominent “skewing” toward the Th2 phenotype, a result similar to that seen in the lacrimal glands of MRL/Mp mice.

In conclusion, the inflammatory lacrimal gland lesions in MRL/lpr and MRL/+ mice appear to be characterized primarily by a Th2 response. The lesions are composed of large numbers of CD4+ T cells staining for IL-4, but relatively few cells staining for IFN-γ. B7-1 positive antigen-presenting cells, which drive the system toward a Th1 response, are only sparsely present, whereas B7-2 positive cells, which drive the system toward a Th2 response, are present in significantly greater numbers. Additional experiments, such as evaluating the amount of mRNA for these cytokines produced in the lacrimal gland, and/or cytokine production by inflammatory cells isolated from the lacrimal gland, and studies blocking either IL-4 or B7-2 with monoclonal antibodies are needed to confirm these results.

References


