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B Cells and Dendritic Cells from Vκ8 Light Chain Transgenic Mice Activate MRL-\textit{lpr}/\textit{gld} CD4\textsuperscript{+} T Cells\textsuperscript{1}

Britte C. Beaudette-Zlatanova,* Tina Ling,* Mark J. Shlomchik,† Ann Marshak-Rothstein,‡ and Ian R. Rifkin²*

Autoreactive CD4\textsuperscript{+} T cells are required for full expression of disease in human systemic lupus erythematosus and in spontaneous murine lupus. However, the Ag specificity of these CD4\textsuperscript{+} T cells remains largely unknown. Rheumatoid factor (RF) B cells function as highly efficient APCs by taking up immune complexes (IC) and presenting IC constituents to T cells. We hypothesized that Ag-specific CD4\textsuperscript{+} T cells in lupus-prone mice could be identified by stimulating the CD4\textsuperscript{+} T cells with RF B cells from AM14 RF BCR transgenic mice pulsed with IC containing lupus-associated autoantibodies and autoantigens. This approach identified several independent T cell lines that proliferated robustly in response to IC-pulsed spleen cells from the AM14 RF BCR transgenic mice. However, these T cells did not recognize an IC constituent. Instead, these T cells recognized a determinant dependent on the inheritance of the transgene-encoded Vκ8 L chain, most likely a neoantigen created by the insertion of the transgene into the genome. Additionally, although the precise nature of the neoantigen is not known, the T cells described in this report may provide a useful tool for examining the role of T cells in the RF autoantibody response. The Journal of Immunology, 2006, 177: 45–52.

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\textsuperscript{3}Abbreviations used in this paper: NZB, New Zealand Black; NZW, New Zealand White; SLE, systemic lupus erythematosus; IC, immune complex; FasL, Fas ligand; ART, autoreactive T cell; RF, rheumatoid factor; PCC, pigeon cytochrome C; KLH, keyhole limpet hemocyanin.

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but on the inheritance of the L chain transgenic locus. To our knowledge, this is the first report of a transgene-modified product stimulating CD4+ T cells.

Materials and Methods

Mice

Mice transgenic for the H chain of the RF BCR (H* mice) (26, 30) were backcrossed to MRL +/- mice and used after the ninth backcross generation. MRL mice transgenic for the Vx8 L chain of the RF BCR (L* mice) (31) were provided by Dr. J. Erikson (Wistar Institute, Philadelphia, PA). Both lines were bred and maintained at the Boston University School of Medicine Laboratory Animal Sciences Center and crossed to produce H+L*, H+L*, H+L*, and H- L* littermates. Inheritance of the transgenes was determined by PCR, and the identity of the H+L* mice was confirmed by flow cytometry using the 4G7 monoclonal anti-Id (26). MRL/MpJ (MRL +/-) mice were obtained from The Jackson Laboratory. Double-mutant MRL- lpr/gld mice were derived from successive crosses of MRL/ MplaFasf8 (MRL-gld) (provided by Dr. C. Sidman, University of Cincinnati, Cincinnati, OH) and MRL/MpJ-Fasf8 (MRL-lpr) as described previously (32). L chain transgenic C57BL/6-Vx8 mice and L chain knockin MRL-Vx8 mice (33) (originally obtained from Dr. M. Weigert, University of Chicago, Chicago, IL) were bred at Yale University School of Medicine. All studies were reviewed and approved by the Institutional Animal Care and Use Committee at Boston University.

Serum samples and Abs

Blood samples collected by tail bleed were allowed to clot at room temperature for 1–2 h, at which time the serum was removed and stored in aliquots at –80°C. The nucleosome-specific IgG2a mAbs PR1-3 and PL2-3 (provided by Dr. M. Monestier, Temple University, Philadelphia, PA) were derived from MRL mice and purified by protein G affinity chromatography (13, 34). The IgA AM14 mAb was isolated from the AM14 L chain transgenic mouse (13, 34). The AM14 mAb and serum IgG were added to the cultures at final concentrations ranging from 25 to 400 μg/ml.

Derivation of CD4+ T cell lines

T-depleted spleen cell suspensions were obtained from MRL AM14 transgenic (RF*) mice by treatment with the mAbs GK1.5 (anti-CD4), 53.6.72 (anti-CD8), and 13-4 (anti-Thy-1.2), followed by rabbit complement (Pel-Freeze Biologicals). The T-depleted spleen cells were preactivated with a CD3 agonist fusion protein and anti-CD8 mAb as previously described (27), and then cultured in 24-well flat-bottom tissue culture plates at a concentration of 2 × 10^6 cells/well. Serum from an MRL-lpr/gld mouse (final concentration 1%) or the anti-nucleosome mAb PR1-3 (final concentration, 7.5 μg/ml) was diluted in complete medium (RPMI 1640, 10% FCS, 20 μM 2-ME, 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin), filter sterilized, and added to the spleen B cells 24 h after the activation step. Both of these conditions were previously described to specifically activate RF+ T cells (27). CD4+ T cells were obtained from the lymph nodes of 5-wk-old MRL-lpr/gld mice by treatment of lymph node cell suspensions with the mAbs 53.6.72 (anti-CD8), 11.5.2 (anti-α1), 14.4.48 (anti-α2), and J11D (anti-HSA), followed by rabbit complement. A total of 2 × 10^4 CD4+ T cells per well was added to the spleen B cells 24 h after addition of the stimulatory serum or anti-nucleosome mAb. Proliferating T cells were enumerated in IL-2 for 10–14 days, and the CD4+ T cells were isolated by magnetic bead purification (Miltenyi Biotec). The purified CD4+ T cells were then restimulated in identical fashion to the initial stimulation; T cells generated from the well originally stimulated with serum were again incubated with serum-pulsed RF+ B cells, and T cells generated from the well originally stimulated with anti-nucleosome mAb were again incubated with anti-nucleosome mAb-pulsed RF+ B cells. This cycle of restimulation was repeated three times to generate the two CD4+ T cell lines called ART.S (serum restimulated) and ART.N (anti-nucleosome mAb restimulated), respectively.

The preparation of T cell hybridomas

The two CD4+ T cell lines ART.S and ART.N were fused with a variant of the AKR thymoma BW5147 using polyethylene glycol (Hybri-Max; Sigma-Aldrich) (35). The resultant hybrids selected in hypoxanthine/aminopterin/8-aminoguanidine medium (Sigma-Aldrich) were tested for their ability to make IL-2 when cultured with spleen cells from MRL mice either expressing (L*) or not expressing (L-) the L chain transgene. T cell hybridomas that produced IL-2 only when cultured with L* spleen cells were identified, and representative clones were chosen for further study. These clones were called ART.N.PG2 (from the fusion between ART.N and BW5147) and ART.S.SB4 and ART.S.SH3 (from the fusion between ART.S and BW5147).

T cell and T cell hybridoma activation by spleen cells

The T-depleted spleen cells were treated with 50 μg/ml mitomycin C (Sigma-Aldrich) at 37°C for 30 min and washed three times. Serum samples, mAbs, or Fab(’), goat anti-mouse IgM (15 μg/ml; Jackson Immunoresearch Laboratories) were added directly to the spleen cells at 5 × 10^6 cells/well in 96-well flat-bottom microtiter plates. Depending on the particular experiment, one or more of the following T cell populations or T cell hybridomas was then added to the splenic cell cultures: 1) freshly isolated CD4+ T cells from the lymph nodes of 5-wk-old MRL-lpr/gld mice; 2) the CD4+ T cell lines ART.S and ART.N, and the pigeon cytochrome C (PCC)-specific H-2 restricted T cell clone AE7 (36); 3) the T cell hybridomas ART.S.SB4 and ART.N.PG2, and the keyhole limpet hemocyanin (KLH)-specific H-2 restricted T cell hybridoma K31H28 (K3) (37). The T cells (1 × 10^5/well) or T cell hybridomas (2 × 10^5/well) were cultured with the treated spleen cells in a total well volume of 200 μl at 5% CO2, 37°C for 24 h, at which time supernatant was removed for measurement of cytokines. In certain experiments, splenic B cells were purified using anti-CD19 magnetic beads (Miltenyi Biotec) and used as the stimulator population.

T cell hybridoma activation by dendritic cells

Myeloid dendritic cells were generated from the bone marrow of L* or L- MRL +/- mice using a modified protocol of that described previously (38). Briefly, bone marrow cells were B cell-depleted using anti-CD19 magnetic beads (Miltenyi Biotec) and cultured in complete medium together with recombinant murine GM-CSF (6.7 ng/ml; BD Pharmingen) and recombinant murine IL-4 (400 pg/ml; R&D Systems). The cells were collected for use in experiments on day 6, at which time ~85% were dendritic cells as judged by CD11c positivity on flow cytometry. Additional purification was performed for certain experiments by positively selecting the CD11c+ cells using a MoFlo cell sorter (DakoCytomation). Dendritic cells were resuspended in complete medium and plated in 96-well flat-bottom microtiter plates at 1 × 10^5 cells/well. The T cell hybridomas ART.N.PG2, ART.S.SB4, and K3 were then added to the plate at 2 × 10^5 cells/well in a total well volume of 200 μl, and the cultures were incubated at 5% CO2, 37°C. After 24 h, supernatant was removed for measurement of IL-2.

Cytokine measurement

IFN-γ and IL-2 in tissue culture supernatants were measured by ELISA according to the manufacturer’s directions (BD Pharmingen).

Flow cytometry

Immunophenotyping of cells from lymph node or from in vitro cultures was performed by flow cytometry as described previously (39). B cells expressing the AM14 H chain together with the Vx8 L chain were detected with the anti-clonotypic mAb 4G7 (26). Analysis was performed using FlowJo software (Tree Star).

Western blot analysis

B cells were purified from the spleens of L* or L- MRL +/- mice using anti-CD19 magnetic beads. Dendritic cells were purified from GM-CSF and IL-4 supplemented cultures of MRL L* and L- bone marrow using anti-CD11c magnetic beads, as described above. Cells were incubated in radioimmunoprecipitation assay buffer containing protease inhibitors (Boehringer Mannheim) for 20 min on ice and centrifuged at 11,750 × g for 10 min at 4°C. Electrophoretic loading buffer was added to the supernatant, and the samples were denatured at 95°C for 5 min. Samples were separated by 10% SDS-PAGE, electrobotted onto a nitrocellulose membrane (Trans Blot Transfer Medium; Bio-Rad), and probed with goat anti-mouse κ conjugated to HRP (Southern Biotechnology Associates). The blots were then stripped and rehybridized using rabbit anti-mouse κ chain (Cell Signaling Technology) followed by goat anti-rabbit IgG-conjugated to HRP (Santa Cruz Biotechnology). Blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce).
Results
Lymph node CD4+ T cells from MRL-lpr/gld mice are activated more strongly by T-depleted spleen from RF+ MRL mice than by T-depleted spleen from RF- MRL mice

Preliminary experiments were performed to test the original hypothesis that RF+ (H+ L+) B cells pulsed with IC containing autoantigen might function as efficient APC for Ag-specific ART. CD4+ T cells were purified from the lymph nodes of 5-wk-old MRL-lpr/gld mice and incubated with T-depleted spleen cells from RF+ mice or RF- (H- L-) littermate controls pretreated with either the anti-nucleosome Ab PL2-3 or with polyclonal anti-IgM F(ab')2. It has been previously demonstrated that PL2-3 forms ICs with chromatin released from the spleen cells in culture and thereby activates the RF+ B cells (27, 28). T cell activation, as evidenced by IFNγ production, was elicited by both RF+ and wild-type spleen but was not seen in the absence of spleen cells (Fig. 1). This is consistent with previous work showing that self-reactive CD4+ T cells from MRL-lpr mice respond to autologous APC in vitro in the absence of exogenous Ag (16, 17). However, the strongest response was elicited by RF+ spleen cells pulsed with chromatin IC, suggesting that there might be additional CD4+ T cell reactivity to components of the chromatin IC.

Certain CD4+ T cell lines from MRL-lpr/gld mice can be activated by spleen cells from MRL mice inheriting the L chain transgene but not by spleen cells from MRL mice not inheriting the L chain transgene

To further study the specificity of the CD4+ T cell response elicited by IC-pulsed RF+ spleen cells, T-depleted spleen cells from RF+ mice were stimulated for 24 h with either an IgG2a anti-nucleosome Ab or with IC containing autoimmune sera. CD4+ T cells purified from the lymph nodes of 5-wk-old MRL-lpr/gld mice were then added to the cultures. Proliferating activated CD4+ T cells were purified from the cultures after 7 days using anti-CD4-coated magnetic beads, expanded in IL-2 in vitro and then restimulated twice more times in identical fashion. Two CD4+ T cell lines from MRL-lpr/gld mice were thus established: one in which the CD4+ T cells had been activated by RF+ spleen cells pulsed with the anti-nucleosome Ab PR1-3 (ART.N), and another in which the CD4+ T cells had been activated by RF+ spleen cells pulsed with stimulatory serum from an MRL-lpr mouse (ART.S). Repeated attempts to generate similar CD4+ T cell lines from MRL+/- mice were unsuccessful.

Following the three identical rounds of restimulation, the specificity of the two CD4+ T cell lines was tested by incubating the cultures with spleen cells from RF+ or littermate control RF- mice that had been pulsed with either anti-IgM F(ab')2, the anti-nucleosome Ab PR1-3, or with autoimmune serum (Table I). IFNγ production by both T cell lines was elicited by RF+ (H+ L+) APCs but not by RF- (H- L-) APCs. The ICs were not required because spleen cells treated with anti-IgM F(ab')2 were as effective in activating the T cell lines as were the IC-pulsed spleen cells. These data indicated that uptake of IC components was not required for activation of the T cell lines.

To determine whether the intact RF+ BCR was required for the stimulatory effect, spleen cells from mice inheriting either the H chain transgene alone (H+ L-) or the L chain transgene alone (H- L+) were included in the analysis (Table I). Unexpectedly, the H+ L- APCs could activate the T cell lines as effectively as the H+ L+ APCs. The failure of H- L- spleen cells to stimulate the T cell lines was not due to any intrinsic defect in Ag presentation because these spleen cells could activate the T cell clone AE7 in the presence of its cognate Ag, PCC (Table I). Thus, the ability of the spleen cells to induce activation of the T cell lines was determined by inheritance of the L chain transgene. TCR Vβ staining demonstrated that each of the T cell lines had skewed toward a particular Vβ, namely Vβ 8.3 in ART.S and Vβ 8.1/2 in ART.N (Fig. 2). This suggested that the transgene-associated epitope specifically activated a limited set of ART.

Table I. APC activation of T cell lines is dependent on inheritance of the L chain transgene

<table>
<thead>
<tr>
<th>T Cell Line</th>
<th>Ligand</th>
<th>H+ L+</th>
<th>H- L-</th>
<th>H+ L-</th>
<th>H+ L-</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART.S</td>
<td>anti-IgM</td>
<td>3.7 ± 0.2</td>
<td>&lt;0.3</td>
<td>15.4 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>PR1.3</td>
<td></td>
<td>4.4 ± 0.6</td>
<td>&lt;0.3</td>
<td>16.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td>3.6 ± 1.2</td>
<td>&lt;0.3</td>
<td>15.4 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>ART.N</td>
<td>anti-IgM</td>
<td>13.3 ± 0.8</td>
<td>&lt;0.3</td>
<td>15.9 ± 0.6</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>PR1.3</td>
<td></td>
<td>12.2 ± 0.9</td>
<td>&lt;0.3</td>
<td>16.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td>10.2 ± 2.2</td>
<td>&lt;0.3</td>
<td>15.5 ± 0.6</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>AE7</td>
<td>None</td>
<td>&lt;0.3</td>
<td>nt</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>PCC</td>
<td>nt</td>
<td>19.8 ± 0.2</td>
<td>nt</td>
<td>16.3 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

* T-depleted spleen cells (APC) from MRL mice that had either inherited (+) or not inherited (−) the H and L chain transgenes were cultured with either anti-IgM, the anti-nucleosome mAb PR1-3, MRL-lpr serum, or PCC. MRL-lpr/gld CD4+ T cell lines were then added to the cultures, and IFNγ-concentration (ng/ml) was measured after 24 h. Data are expressed as the mean ± SD of triplicate cultures. A representative example of two to four experiments is shown. nt, Not tested.

FIGURE 1. MRL-lpr/gld CD4+ T cells are preferentially activated by RF+ APC pulsed with a nucleosome-specific mAb. T-depleted spleen cells from MRL RF+ (H+ L+) and RF- (H- L-) mice were stimulated with 15 μg/ml anti-IgM or 15 μg/ml anti-nucleosome mAb PL2-3. MRL-lpr/gld CD4+ lymph node T cells were then added to the spleen cell cultures, or to wells without spleen cells (no APC) coated or not coated with anti-CD3 mAb. The cultures were incubated for a further 24 h, and IFNγ in the supernatants was measured by ELISA. Data are expressed as the mean ± SD of triplicate cultures. A representative example of three experiments is shown.

FIGURE 2. The MRL-lpr/gld CD4+ T cell lines ART.S and ART.N are skewed with regard to Vβ expression. The CD4+ T cell lines ART.S and ART.N were analyzed after the third round of restimulation by flow cytometry. MRL+/- lymph node cells were used as a control.
$T$ cell hybridoma analysis confirms that the $T$ cell response is MHC restricted and dependent on the inheritance of the $L$ chain transgene locus

$T$ cell hybridomas were made from the two CD4$^+$ $T$ cell lines to analyze the specificity of the response at a clonal level. These $T$ cell hybridomas were initially screened for reactivity to $T$-depleted spleen cells from mice bearing the $L$ chain transgene by measuring IL-2 production. From the fusion of the ART.N $T$ cell line with the BW5147 tumor cell line, five $T$ cell hybridomas were obtained. The most strongly reactive of these, called ART.N.PG2, expressed $V_L^8$1/2, the dominant $V_L$ specificity of the ART.N $T$ cell line. From the fusion of the ART.S $T$ cell line with the BW5147 tumor cell line, four $T$ cell hybridomas were obtained. Two of these were strongly reactive with spleen cells from mice bearing the $L$ chain transgene. One, designated ART.S.SH3, expressed $V_L^8$3, the dominant $V_L$ specificity of the ART.S $T$ cell line. A second, designated ART.S.SB4, surprisingly did not express $V_L^8$3. Although clearly expressing a $V_L$ TCR by pan-$V_L$ staining, the precise $V_L$ used by ART.S.SB4 remains unidentified; reagents specific for $V_L^2$, 3, 4, 6, 7, 8, 1/2, 8.3, 10, 13, and 14 failed to stain these cells. The $T$ cell hybridomas ART.N.PG2 and ART.S.SB4 were selected for further study as representative examples of hybridomas from the $T$ cell lines ART.N and ART.S, respectively.

To confirm that the reactivity of the selected $T$ cell hybridomas was the same as the $T$ cell lines from which they were derived, ART.S.SB4 and ART.N.PG2 were incubated with $T$-depleted spleen cells from $H^L^L$, $H^L^L$, $H^L^L$, and $H^L$ mice pulsed with either the anti-nucleosome Ab PR1-3 or with anti-IgM F(ab')$_2$. As expected, IL-2 production by ART.S.SB4 and ART.N.PG2 was seen only in cultures stimulated with spleen cells from mice inheriting the $L$ chain transgene ($H^L^L$, $H^L^L$, or $H^L^L$) (Table II). Also as expected, IL-2 production was not dependent on the nature of the stimulus (anti-IgM or anti-nucleosome Ab), confirming that it was inheritance of the $L$ chain transgene locus per se that conferred reactivity. Activation of the hybridomas was due to non-specific factors in the spleen cell cultures because the $KLH$-specific $T$ cell hybridoma K3 did not produce IL-2 in the absence of its cognate Ag.

To determine whether the $T$ cell response was MHC restricted, spleen cells were obtained from mice in which the $L$ chain transgene had been bred onto the C57BL/6 genetic background. L$^+$ C57BL/6 cells did not stimulate the $T$ cell hybridomas to make IL-2, thereby demonstrating the MHC dependence of the $T$ cell response (Table III).

Table II. $T$ cell hybridoma activation is dependent on the inheritance of the $L$ chain transgene

<table>
<thead>
<tr>
<th>T Hyridoma</th>
<th>Ligand</th>
<th>$H^L^L$</th>
<th>$H^L$</th>
<th>$H^L^L$</th>
<th>$H^L^L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART.S.SB4</td>
<td>anti-IgM</td>
<td>855 ± 10</td>
<td>&lt;150</td>
<td>3000 ± 55</td>
<td>&lt;150</td>
</tr>
<tr>
<td>PR1-3</td>
<td></td>
<td>559 ± 60</td>
<td>&lt;150</td>
<td>3061 ± 34</td>
<td>&lt;150</td>
</tr>
<tr>
<td>ART.N.PG2</td>
<td>anti-IgM</td>
<td>617 ± 46</td>
<td>&lt;150</td>
<td>1306 ± 139</td>
<td>&lt;150</td>
</tr>
<tr>
<td>PR1-3</td>
<td></td>
<td>705 ± 46</td>
<td>&lt;150</td>
<td>2083 ± 49</td>
<td>&lt;150</td>
</tr>
<tr>
<td>K3</td>
<td>None</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>nt</td>
<td>nt</td>
</tr>
</tbody>
</table>

$KLH$ 380 ± 26 440 ± 80  nt  nt  284 ± 24

$^a$ $T$-depleted spleen cells (APC) were prepared from mice that had either inherited ($L^+$) or not inherited ($L^-$) the $L$ chain transgene (Tg), or from $L$ chain knockin mice ($L^{KI}$). Two different sources of V$\kappa$L chain, either AM14 IgA or IgG from the sera of $L$ chain transgenic mice ($L$ chain IgG) were added as shown. $T$ cell hybridomas were then added to the cultures, and IL-2 production (pg/ml) was measured after 24 h. Data are representative of two to three experiments and is expressed as the mean ± SD of triplicate cultures. nt, Not tested.

Table III. $T$ cell hybridoma activation is not due to recognition of a $L$ chain epitope but is MHC restricted

<table>
<thead>
<tr>
<th>APC</th>
<th>Tg/KI</th>
<th>MHC</th>
<th>$H^L^L$</th>
<th>$H^L$</th>
<th>$H^L^L$</th>
<th>$H^L^L$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ART.S.SB4</td>
<td>ART.N.PG2</td>
<td>K3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$H^L^L$ k</td>
<td>None</td>
<td>783 ± 163</td>
<td>596 ± 58</td>
<td>&lt;150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>None</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td></td>
<td></td>
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<tr>
<td>$H^L$ k</td>
<td>None</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM 14 IgA</td>
<td>L chain IgG</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$KLH$</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>594 ± 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H $L^{KI}$ k</td>
<td>None</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$KLH$</td>
<td>nt</td>
<td>nt</td>
<td>284 ± 24</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

$^a$ $T$-depleted spleen cells (APC) were prepared from mice that had either inherited ($L^+$) or not inherited ($L^-$) the $L$ chain transgene (Tg), or from $L$ chain knockin mice ($L^{KI}$). The $T$ cell response was not directed against a $L$ chain epitope

At this point, the most likely interpretation of the data was that the CD4$^+$ $T$ cell lines recognized a component of the transgene-encoded $L$ chain presented by the MRL B cells. Autoantibody idiotopes have been shown to activate ART in other murine models of SLE (22, 23), and it was possible that a similar reactivity was present in MRL mice. We used two approaches to determine whether or not the reactivity to spleen cells from the $L$ chain transgenic mice was due to recognition of a peptide from the Ig $L$ chain.

The first approach was to determine whether MRL $H^L^L$ $APCs$ pulsed with Ig containing the transgene-encoded $L$ chain could activate the $T$ cell hybridomas ART.S.SB4 and ART.N.PG2. This Ig was obtained by 1) purifying Ig from the $B$ cell hybridoma AM14 (40), which uses a $L$ chain identical with that of the transgene-encoded $L$ chain, except for a single amino acid substitution at position 29 (Pro instead of Gln) (26), and 2) purifying IgG, which should contain an appreciable amount of the transgene-encoded $L$ chain, from the sera of MRL $L^+$ transgenic mice. Unexpectedly, MRL spleen cells pulsed with either of these sources of transgene-encoded $L$ chain failed to activate the $T$ cell hybridomas ART.S.SB4 and ART.N.PG2 (Table III), although $KLH$-pulsed spleen cells could activate the $KLH$-specific $T$ cell hybridoma K3.

The second and more definitive approach was to use a $L$ chain knockin mouse. The $L$ chain knockin mouse was made with the same VDJ sequence as the $L$ chain transgenic mouse (31, 33). Spleen cells from the knockin mice completely failed to induce hybridoma IL-2 production, whereas spleen cells from the $L$ chain transgenic mice effectively induced IL-2 production (Table III). The knockin spleen cells effectively activated the $KLH$-specific $T$ cell hybridoma K3. Although we do not have a reagent specific for V$\kappa$8, we believe that the inability of the spleen cells from the knockin mice to activate the hybridomas was not due to impaired V$\kappa$8 $L$ chain production. When crossed to the AM14 $H$ chain, $B$ cells from the transgenic and knockin V$\kappa$8 mice express comparable levels of AM14 $H$ chain/V$\kappa$8 surface IgM as detected with the anti-clonotype mAb 4G7 (Fig. 3) (26). These results indicate, that contrary to expectations, the CD4$^+$ $T$ cell reactivity to spleen cells from the $L$ chain transgenic mice was not due to recognition of a peptide from the Ig $L$ chain itself but rather to the presentation of a non-$L$ chain Ag(s) presumably arising as a consequence of insertion of the $L$ chain transgene into the genome.
The T cell hybridomas ART.SSB4 and ART.N.PG2 respond to L chain transgenic dendritic cells as well as B cells

If, as suggested by the above studies, it was not the L chain itself that was being presented but rather a non-L chain Ag(s), then it might be expected that APC other than B cells would be capable of efficient T cell activation. To investigate this possibility, spleen cells from H⁺L⁺ mice were separated into B cell-enriched and B cell-depleted fractions using anti-CD19-coated magnetic beads. The Id-specific Ab 4G7 was used to identify B cells expressing the L chain transgenic and H chain combination (26). At least 80% of the B cells were purified from the spleens of wild-type (WT) mice, whereas only 5% of cells in the B cell-depleted spleen cell fraction from the H⁺L⁺ mice expressed this combination, as determined by flow cytometry (Fig. 4A). The B cell-enriched fraction was then compared with the B cell-depleted fraction for their ability to activate the T cell hybridomas ART.SSB4 and ART.N.PG2 (Fig. 4B). Both fractions elicited comparable cytokine production. These data indicate that non-B cells in the spleen cells from L chain transgenic mice could induce T cell activation. However, it was not possible to exclude the possibility that the residual 5% of L chain transgenic B cells in the B cell-depleted fraction were contributing to this activation.

To address this concern, and also to identify at least one specific non-B cell population capable of inducing T cell activation, B cell-depleted bone marrow cells were cultured for 6 days in the presence of GM-CSF and IL-4. After 6 days in culture, ~85% of the cells were dendritic cells as evidenced by CD11c positivity (Fig. 5A). Importantly, no B cells were present. Dendritic cell-enriched cultures from L chain transgenic mice, but not wild-type mice, were able to activate the T cell hybridomas ART.SSB4 and ART.N.PG2 (Fig. 5B). CD11c⁺ cells derived from comparable cultures were further purified using a cell sorter (Fig. 5C). These CD11c⁺ dendritic cells were able to activate the T cell hybridomas ART.SSB4 and ART.N.PG2, indicating that dendritic cells alone were sufficient for activation (Fig. 5D). The activation induced by the dendritic cells was due to non-specific effects because the KLH-specific T cell hybridoma K3 was not activated (Fig. 5D). These experiments demonstrated that a non-B cell population from L chain transgenic mice was able to activate ART.SSB4 and ART.N.PG2.

Dendritic cells from L chain transgenic mice do not produce κ L chain protein

One possible explanation for activation of the T cell hybridomas ART.SSB4 and ART.N.PG2 by the L chain dendritic cells was that the transgene insertion might have resulted in the abnormal expression of the Vκ8 L chain protein in the dendritic cells. To test this, we compared the amount of κ L chain protein in the L chain transgenic B cells and dendritic cells by Western blotting. We used a detecting Ab that recognizes all mouse κ L chains, because an Ab specific for the Vκ8 L chain protein is not available. As expected, large amounts of κ L chain protein were present in B cells from both L chain transgenic and wild-type mice (Fig. 6). The large majority of L chains in normal mice are κ with 10% or less being λ (41), so it was not surprising that no appreciable difference in the amount of κ L chain protein was seen between the transgenic and wild-type B cells. In contrast, no κ L chain protein was detected in either transgenic or wild-type dendritic cells (Fig. 6). Thus, T cell hybridoma activation by the L chain transgenic dendritic cells is not due to abnormal L chain protein production by the dendritic cells.

Discussion

CD4⁺ T cell lines, derived from unmanipulated MRL-lpr/gld mice, can be activated by B cells and dendritic cells transgenic for
The functionally rearranged V_{H}9260 L chain transgene used in these experiments (Ga V_{H}9260) was originally identified as the gene encoding the Ab L chain most frequently generated by BALB/c mice in the primary response to the antigenic site, Sb, of the influenza virus A/PR/8/34 hemagglutinin molecule (31). The transgene contains all the necessary elements required for normal regulated transcription (31). It was used as the L chain component in the initial development of the AM14 RF transgenic mouse model because it is almost identical with the V_{H}9260 used by the original MRL-lpr-derived AM14 RF B cell hybridoma. The binding activity of the AM14 H/Ga V_{H}9260 combination is indistinguishable from the AM14 RF (26).

What can account for the observation that CD4^{+} T cells from unmanipulated MRL-lpr/gld mice can be activated by B cells and dendritic cells from the L chain transgenic mice? Other investigators have described ART that recognize self-idiotope peptides, both in humans with SLE and in murine SLE models (22, 23, 42).

However, there are a number of lines of evidence indicating that the T cell response demonstrated in this report is not directed against the transgenic L chain protein per se. First, and most importantly, the identical VDJ sequence was used to make both the L chain transgenic and the L chain knockin mice (31, 33), and only cells from the transgenic line can stimulate our T cell lines and hybridomas. Therefore, unless the L chain gene product is somehow processed or presented differently in the knockin as compared with the transgenic mice, it would be expected that spleen cells from both the transgenic and knockin mice would induce similar degrees of T cell activation. However, this is not what was observed. Despite the fact that an anti-clonotypic Ab specific for a combined H chain/V_{H}9260 L chain idiotope stained both AM14 L chain transgenic and AM14 L chain knockin B cells to a comparable extent, only the transgenic spleen cells induced T cell activation. Second, the addition of purified Ig containing the transgene-encoded L chain to wild-type APC failed to elicit T cell activation.

FIGURE 5. Dendritic cells, in the absence of B cells, activate the T cell hybridomas ART.S.SB4 and ART.N.PG2. A, Bone marrow cells from MRL H^{+} L^{+} and MRL H^{+} L^{-} mice were B cell depleted and cultured in GM-CSF and IL-4 for 6 days. Flow cytometry of cells from the H^{+} L^{+} mice is shown (solid line, unstained cells; dotted line, stained cells) but was similar for both genotypes. B, These dendritic cell-enriched, B cell-negative cells were cultured with the T cell hybridomas ART.S.SB4, ART.N.PG2, and the KLH-specific, T cell hybridoma K3. IL-2 production after 24 h was measured by ELISA. Data are expressed as the mean ± SD of triplicate cultures. A representative example of five experiments is shown. C, Bone marrow cells from MRL H^{+} L^{+} mice were B cell depleted and cultured in GM-CSF and IL-4 for 6 days. CD11c-positive, IgM-negative cells were then purified by cell sorter, the gated cells (in lower right quadrant) being those used in subsequent assays. D, CD11c^{+} sorted purified dendritic cells from MRL H^{+} L^{+} mice and T-depleted MRL H^{+} L^{+} and H^{+} L^{-} spleen cells were cultured with the T cell hybridomas ART.S.SB4, ART.N.PG2, and K3. IL-2 production after 24 h was measured by ELISA. Data are expressed as the mean ± SD of triplicate cultures.
Finally, despite the fact that the precise nature of the neoeu antigen(s) is not known, the T cells that recognize it could prove to be useful experimental tools. AM14 (H\(^{L^+}\)) transgenic mice have provided a highly relevant model to study the regulation of autoreactive B cells (26). However, one limitation of the model has been the unavailability of T cells that specifically recognize the H\(^{L^+}\) transgenic B cells. The use of the T cells described in this report should facilitate studies examining the role of T cells in the generation of the RF autoantibody response.

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Disclosures

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References

11. Peng, S. L., M. P. Madaios, A. C. Hayday, and J. Craft. 1996. Propagation and expression of costimulatory molecules. Chromatin IC activate B cells for T cell activation. Third, dendritic cells would not be expected to transcribe a L chain transgene driven off the B cell promoter used in this model (native V\(\kappa\) and \(\kappa\) enhancer), and indeed we found no evidence of \(\kappa\) L chain protein production by the dendritic cells. Nevertheless, dendritic cells from the L chain transgenic mice were found to elicit strong T cell responses. The dendritic cells were generated in vitro from bone marrow rather than being harvested directly from the spleen to preclude the possibility of exogenous uptake of L chain as would be possible with splenic dendritic cells. Thus, recognition of an Ig peptide is an unlikely cause of the T cell reactivity seen in our experiments.

Transgenic mice have been invaluable in the study of many aspects of modern biology. Ig transgenic mice have been used for more than a decade to study gene rearrangements, allelic exclusion, tolerance, and autoimmunity during B cell development (43). Generation of transgenic mice by microinjection of cloned DNA into the pronucleus of a fertilized mouse egg results in the random integration of the exogenous DNA into the host chromosomes (44). Frequently, rearrangements, deletions, duplications, or translocations occur at the insertion sites (45). These mutations will be detected only if they cause a recognizable mutant phenotype (44). In contrast to AM14 H chain homozygous mice, neither L chain transgenic nor L chain knockin mice have an obvious developmental defect. Even so, mutations might be created that result in more subtle alterations in endogenous protein structure or function, but which do not cause an obvious mutant phenotype. Immunogenic neoantigens could be created by this process. Therefore, the most likely explanation for our findings is that the site(s) of insertion of the L chain transgene into the genome resulted in the formation of such a neoantigen(s), which could then be presented by both dendritic cells and B cells to induce CD4+ T cell activation. Alternatively, another mechanism, such as an alteration of the Ag-processing machinery by the transgene, might be operative. Experiments to distinguish between these possibilities are currently in progress.

It is interesting that the unmanipulated naïve CD4+ T cells from MRL-lpr/gld mice were initially more strongly activated by H\(^{L^+}\) spleen cells cultured with anti-nucleosome mAb (chromatin IC) than by H\(^{L^+}\) spleen cells cultured with F(ab')\(_2\) anti-IgM. One possible explanation is that the chromatin ICs confer better Ag-presenting properties to the spleen cells by inducing stronger expression of costimulatory molecules. Chromatin IC activate B cells by coengagement of the BCR and TLR9 (28), and TLR activation of APCs has been shown to play a critical role in T cell-dependent models of autoimmune disease (46, 47).

**FIGURE 6.** Dendritic cells from L chain transgenic mice do not express \(\kappa\) L chain protein. B cells were purified from the spleens of MRL mice that had either inherited (L\(^{L^+}\)) or not inherited (L\(^{L^-}\)) the L chain transgene. Dendritic cells (DCs) were purified from GM-CSF and IL-4-supplemented cultures of L\(^{L^-}\) bone marrow. Expression of \(\kappa\) L chain protein was analyzed by Western blotting. The same blot was reprobed for \(\beta\) actin to demonstrate equal protein loading. A representative example of three experiments is shown.


