L Chain Allelic Inclusion Does Not Increase Autoreactivity in Lupus-Prone New Zealand Black/New Zealand White Mice

Efi Makdasi and Dan Eilat

*J Immunol* 2013; 190:1472-1480; Prepublished online 14 January 2013;
doi: 10.4049/jimmunol.1202331
http://www.jimmunol.org/content/190/4/1472

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/01/15/jimmunol.120233.1.DC1

**References**

This article cites 39 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/190/4/1472.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
L Chain Allelic Inclusion Does Not Increase Autoreactivity in Lupus-Prone New Zealand Black/New Zealand White Mice

Efi Makdasi and Dan Eilat

L chain allelic inclusion has been proposed as a B cell tolerance mechanism in addition to clonal deletion, clonal anergy, and receptor editing. It is said to rescue autoreactive B cells from elimination by diluting out the self-reactive BCR through the expression of a second innocuous L chain. In autoimmune animals, such as lupus-prone mice, allelically included B cells could be activated and produce pathogenic autoantibodies. We have previously shown that anti-DNA hybridomas from diseased New Zealand Black/New Zealand White F1 mice exhibit nearly perfect allelic exclusion. In the current study, we have analyzed single B cells from these and from nonautoimmune mice. In addition, we have cloned and expressed the Ig variable regions of several L chain–included B cells in cell culture. We find that although the number of L chain-included B cells increases as a result of receptor editing, the majority of such cells do not retain an autoreactive HxL chain combination and, therefore, allelic inclusion in itself does not serve as a B cell tolerance mechanism in these autoimmune mice. The Journal of Immunology, 2013, 190: 1472–1480.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

The online version of this article contains supplemental material.

Abbreviations used in this article: ANA, antinuclear Ab; GC, germinal center; MZ, marginal zone; NZB, New Zealand Black; NZW, New Zealand White; SLE, systemic lupus erythematosus.

E-mail address: eilatd@cc.huji.ac.il
Address correspondence and reprint requests to Prof. Dan Eilat, Department of Medicine, Hadassah University Hospital, Ein Kerem, Jerusalem 91120, Israel

Department of Medicine, Hadassah University Hospital, Faculty of Medicine, Hebrew University, Jerusalem 91120, Israel
Received for publication August 20, 2012. Accepted for publication December 12, 2012.
This work was supported by the Israel Science Foundation, founded by the Academy of Science and Humanities; E.M. was supported by a fellowship from the ISEF Foundation.
Address correspondence and reprint requests to Prof. Dan Eilat, Department of Medicine, Hadassah University Hospital, Ein Kerem, Jerusalem 91120, Israel. E-mail address: eilatd@cc.huji.ac.il
The online version of this article contains supplemental material.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1202331
Materials and Methods

Mice

All mice were maintained at the specific pathogen-free animal facility of the Hebrew University (Jerusalem, Israel). The hCk knockin mice were produced by Casellas and coworkers (15) and backcrossed to the BALB/c background by Dr. Y. Bergman in Jerusalem. NZB and NZW mice were purchased from Harlan (Oxon, U.K.). The hCk/mCk NZB/NZW and D42H NZB/NZW mice were obtained by backcrossing hCk BALB/c onto the NZW genetic background for eight generations, and then mating the hCk NZW male mice with wild-type or D42H knockin, female NZB mice. The NZB/NZW and D42H NZB/NZW mice used throughout this study were 6–10 mo-old females and had high anti-DNA titers, compatible with active disease. The normal mouse controls were 3- to 4-mo-old females. All mouse studies were reviewed and approved by the Ethics Committee of the Authority for Animal Facilities, The Hebrew University.

Abs

The following reagents were used for FACS analysis and sorting: rat anti-mouse CD138-biotin, streptavidin PE-Cy 5, rat anti-mouse GL7-FITC (BD Pharmingen, San Diego, CA); rat anti-mouse CD23-PE (eBioscience, San Diego, CA); goat anti-mouse IgM + IgG (H + L) (Jackson Immune Research); HRP–rat anti-mouse IgG, HRP–goat anti-mouse IgM, HRP–goat anti-mouse IgG Fc, HRP–goat anti-mouse IgM + IgG (H + L), goat anti-human IgG Fc, donkey anti-mouse IgM, donkey anti-mouse IgG, HRP–anti-mouse IgG, HRP–goat anti-mouse IgG Fc, HRP–goat anti-idiotype (18); goat anti-mouse IgM + IgG (H + L); goat anti-mouse IgG (ICL, Newberg, OR).

Experimental procedures

Hybridoma production, flow cytometric analysis, reverse transcription, sequence determinations, and DNA-binding tests were performed as described previously (18).

ANA was performed with a Kallestad HEp-2 Cell Line Substrate Kit (Bio-Rad, Redmond, WA).

The hCk/mCk-expressing mice were identified by PCR of tail DNA using human + mouse constant κ forward primer 5′-ATAAACCTGGGTGAA-TCTCTGTCTG-3′ and the following constant κ reverse primers: mouse 5′-ACGCCATTTTGTCGTTCACTGCCA-3′ or human 5′-GAGTTACCAGGA-TGGAGGGCGTTA-3′. PCR was performed at 94˚C for 5 min, followed by 30 cycles at 94˚C for 30 s, 55˚C for 30 s, 72˚C for 45 s, and finally at 72˚C for 5 min.

Sorting of single cells and CDNA synthesis were carried out as described by Wintsch and Bettelheim (16). The first single-cell PCR reaction was performed in 96-well plates containing 2 μl cDNA, 20 μl DreamTaq Mix ×2 (Fermentas, Burlington, ON, Canada), and 200 nM of the following primers: forward Vκ (FW3) primer: 5′-GGCTGCTGCTTATGCAGGAGTGGGCT-GATCT-GWGRAC-3′; and the Ck reverse primers: human 5′-CCTCCTGGGAGTTACGT TAGGT-3′, mouse 5′-CTCCTGCTTACGATGGTAATT-3′, or mouse 5′-CTACCTGCTTACAGGAATTT-3′. For mouse Igα amplification, the forward VA1/2 primer 5′-CAGTACATCTCACTTCTGCCTCA-3′ and the reverse Ck internal primer 5′-GAGCTTCTTACAGGAGGTCCG-3′ were used.

Cloning and expression of Ig genes from single B cells were carried out as described by Tiller et al. (19).

Results

FACS sorting of human/mouse Cκ B cells

Normal mouse hCk/mCk-expressing mice were obtained by mating homozygous human Cκ knockin BALB/c mice with wild-type C57BL/6 (B6) mice. FACS analysis of B220+ gated B cells showed (Fig. 1A) that the anti-mouse and anti-human Cκ reagents were highly specific and that B cells from hCk/mCk (B6xBALB/c) F1 mice expressed the human and mouse Cκ L chain in nearly equal amounts. Approximately 4% of the B cells in these mice were double expressors of human and mouse Cκ L chains. The single- and double-producer populations could be sorted to near homogeneity (Fig. 1A). RT-PCR analysis of single B cells, obtained from the sorted double-producer population showed that the great majority (∼80%) of these cells expressed in-frame human and mouse Cκ L chains (Fig. 1B, 1C); however, ∼20% of these cells expressed only a single (human or mouse) L chain, probably owing to a contamination by overlapping cell populations. To further test that the two L chains were produced by a single B cell and not by two separate cells, equal numbers of hCk- and mCk-producing B cells were mixed and FACS analyzed (Fig. 1D). Only a negligible background of cells in the upper right quadrant could be observed; furthermore, 200 individual B cells, picked at random from this mixture, were analyzed by RT-PCR and showed either mCk or hCk L chain expression, but not both (data not shown). These experiments show that expression of both mouse and human Cκ L chains by a single-cell FACS analysis is indeed due to allelic inclusion.

Single-cell and hybridoma analysis of L chain–included B cells from diseased NZB/NZW mice

To study allelic inclusion in wild-type and anti-DNA transgenic NZB/NZW mice, the hCk knockin normal mice were backcrossed for eight generations onto the genetic background of NZW mice. The homozygous hCk male mice were then mated with either wild-type or D42H knockin (18) NZB female mice. The NZB/NZW F1 female offspring were followed for 7–8 mo until their anti-DNA titers were compatible with a fully developed lupus-like disease (20). The hCκ L chain had no apparent influence over disease development or survival of these mice, compared with unmanipulated NZB/NZW mice. FACS analysis of splenic B cells from hCk/mCk NZB/NZW mice (Fig. 1E) showed a picture similar to that of normal hCk/mCk mice, with similar proportions of allelically included cells. However, owing to a reduced BCR density as a consequence of clonal anergy (6, 21), the three B cell populations in the diseased female NZB/NZW mice were less well separated from one another compared with the same populations in normal mice (Fig. 1E); consequently, only ∼30–40% of the highest staining cells in the upper right quadrant were shown to be true double hCk/mCk producers in these mice. In addition, it has previously been shown that a large proportion of L chain–included B cells are found in the allelically excluded populations because of an unequal expression of the two L chains by the same cell (11, 15). Therefore, in subsequent experiments, we analyzed single cells from whole B220+ B cell populations rather than from FACS-sorted upper right quadrant, double-producer populations.

Hybridoma analysis was carried out in parallel with the single-cell study. Allelically included hCk/mCk hybridomas were identified by intracellular staining with anti-hCκ and anti-mCκ reagents (15). Each double L chain–producer hybridoma was recloned to eliminate any contaminating single-producer hybridomas.
Table I and Fig. 2 summarize the results of single-cell analysis of B cells derived from the spleens of hCk/mCk normal mice, wild-type NZB/NZW mice, and anti-DNA D42H knockin NZB/NZW mice. The percentages of κ-included B220-positive cells were similar in the normal and lupus-prone animals (6–10%, Fig. 2A, Table I). The κ/l double-producer B cells were nearly absent in all of the tested mice (Table I). D42 transgenic NZB/NZW mice had a higher proportion of L chain–included B cells (13–40%), apparently because of their higher receptor editing activity (22).

Marginal zone (MZ) B cells (B220+ IgMhi CD23+2) had a higher proportion of κ-included B cells than total B220-positive or germinal center [(GC) B220+ GL-7+] B cells (Fig. 2B), in agreement with previous results (9, 15).

To examine further the correlation between L chain allelic inclusion and receptor editing, single B cells from the three groups of mice were tested for Jκ usage. Nonediting B cells mostly use Jκ1 or Jκ2 for κ-chain usage, whereas editing B cells often shift to Jκ4 or Jκ5 usage (22, 23). Fig. 2C shows that normal mice and wild-type NZB/NZW mice had a similar profile of Jκ usage, with a higher proportion of Jκ1 + Jκ2 compared with Jκ4 + Jκ5 usage and a low level of L chain allelic inclusion. In contrast, the higher double L chain–producer B cells from D42H transgenic mice had a higher percentage of Jκ4 + Jκ5 usage, indicating a higher level of L chain receptor editing in these mice (Fig. 2C).

Although our previous hybridoma study (18) was limited to anti-DNA hybridomas and to a small number of L chains, it was still puzzling that the level of allelic inclusion was much lower than that observed in this study, using single sorted B cells. We therefore compared the present analysis to hybridomas obtained from activated B cells (i.e., spontaneous hybridomas) and to LPS-induced hybridomas, presumably derived from nonactivated B cells (20, 24, 25). Of interest, the level of L chain allelic inclusion in normal mice was several-fold higher in the LPS hybridomas than in the spontaneous hybridomas and similar to the level of allelic inclusion in the single-cell analysis (Fig. 2D). This observation suggests that most L chain–included B cells are not activated, as would be expected from their lower effective BCR density and mixed Ag specificity. Furthermore, this situation is very similar in diseased, lupus-prone NZB/NZW mice (Fig. 2D, Table II). This finding is also supported by the low percentage of L chain–included hybridomas among both spontaneous and LPS-activated anti-DNA hybridomas from hCk/mCk NZB/NZW mice (Table II). In anti-DNA transgenic hCk/mCk D42H NZB/NZW mice, the percentage of anti-DNA hybridomas from total hybridomas was three to four times higher than in wild-type NZB/NZW mice.
NZW mice, but the fraction of L chain–included hybridomas in this population was only 1–2% (Table II), in agreement with our previous study (18).

Autoreactivity of L chain–included B cells from diseased NZB/NZW mice

The major question in this study is whether L chain allelic inclusion can be regarded as a B cell tolerance mechanism that serves to rescue autoreactive B cells from elimination or whether it is a byproduct of L chain rearrangement, particularly secondary rearrangement, and has little or no consequences for immune tolerance.

As illustrated in Fig. 3, in the first case, one would expect that the included peripheral B cells should retain one autoreactive and one nonautoreactive HxL combination, as was found in several transgenic mice expressing autoantibodies (9–11, 13), whereas in the second case, most included B cells would be nonautoreactive.

We have tested these predictions in two ways: In the first, we compared the antinuclear Ab (ANA) activity of L chain–included hybridomas with the activity of allelically excluded hybridomas. In the second, we cloned the rearranged V-region genes from the H chain and the two L chains, derived from individual included B cells, as well as those of H and L chains from individual excluded B cells; we then expressed the cloned V(D)J regions as human IgG1 in human embryonic kidney cells in culture and tested each purified HxL combination for ANA specificity.

Fig. 4A shows the ANA patterns that were observed. They included homogeneous, speckled, nuclear membrane, and nucleolar patterns, similar to familiar ANA patterns observed with hybridomas from diseased female NZB/NZW mice. The percentage of ANA-positive supernatants, belonging to L chain–included hybridomas from wild-type hC<sub>k</sub>/mC<sub>k</sub> NZB/NZW mice, was very low (13%) and identical to that of excluded hybridomas from the same diseased mice (Fig. 4B). For comparison, included hybridomas from normal, (B6xBALB/c) F1 mice had a much lower ANA reactivity (6%). Most importantly, the expressed H or L chain products derived from the single B cells also gave a very low percentage of ANA-positive Abs (Table III, Supplemental Tables I, II, and Fig. 4C) that was similar for included and excluded B cells. About 50% of the single cell–expressed Abs showed various ANA-negative, Hep-2 cell fluorescent patterns (totally negative, cytoplasmic, filamentous, and nonspecific; Supplemental Tables I, II) that were equally distributed between L chain–included and –excluded B cells. In hybridomas, the extent of nonANA binding was lower than in single cell–expressed Abs (27% in B6xBALB/c and 23% in NZB/NZW included hybridomas; data not shown), presumably owing to the presence of neutralizing serum proteins in the hybridoma supernatants. These results suggest that L chain–included B cells are generally not autoreactive. This conclusion is strongly supported by the anti-DNA hybridoma results (Table II).

Discussion

The observed frequencies of IgH and IgL allelic inclusion among peripheral B cells were initially estimated at <0.1% and 2%, respectively (26, 27). However, L chain receptor editing may in-

### Table I. Allelic inclusion in single B cells from hC<sub>k</sub>/mC<sub>k</sub> mice

<table>
<thead>
<tr>
<th>Mouse/C&lt;sub&gt;k&lt;/sub&gt;/mC&lt;sub&gt;k&lt;/sub&gt; Mouse</th>
<th>B Cell Population</th>
<th>Mouse C&lt;sub&gt;k&lt;/sub&gt; (%)</th>
<th>Human C&lt;sub&gt;k&lt;/sub&gt; (%)</th>
<th>Lambda (%)</th>
<th>Double hC&lt;sub&gt;k&lt;/sub&gt;/mC&lt;sub&gt;k&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6xBALB/c no. 1</td>
<td>B220</td>
<td>37/70</td>
<td>29/70</td>
<td>ND</td>
<td>4/70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(53)</td>
<td>(41)</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>B6xBALB/c no. 2</td>
<td>B220</td>
<td>50/84</td>
<td>28/84</td>
<td>ND</td>
<td>6/84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(60)</td>
<td>(33)</td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>B/W no. 1</td>
<td>B220</td>
<td>43/93</td>
<td>39/93</td>
<td>2/93</td>
<td>9/93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(46.2)</td>
<td>(42)</td>
<td>(2.15)</td>
<td>(9.6)</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>48/92</td>
<td>25/92</td>
<td>6/92</td>
<td>13/92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(52)</td>
<td>(27)</td>
<td>(6.5)</td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>41/93</td>
<td>38/93</td>
<td>5/93</td>
<td>9/93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(44)</td>
<td>(40)</td>
<td>(5.5)</td>
<td>(9.5)</td>
</tr>
<tr>
<td>B/W no. 2</td>
<td>B220</td>
<td>40/91</td>
<td>44/91</td>
<td>1/91</td>
<td>6/91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(44)</td>
<td>(48.4)</td>
<td>(1.2)</td>
<td>(6.5)</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>36/75</td>
<td>29/75</td>
<td>2/75</td>
<td>8/75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(48)</td>
<td>(38.6)</td>
<td>(2.6)</td>
<td>(10.7)</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>40/84</td>
<td>38/84</td>
<td>1/84</td>
<td>7/84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(47.6)</td>
<td>(42.8)</td>
<td>(1.3)</td>
<td>(8.3)</td>
</tr>
<tr>
<td>B/W no. 3</td>
<td>B220</td>
<td>35/84</td>
<td>42/84</td>
<td>ND</td>
<td>7/84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(42)</td>
<td>(50)</td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>26/53</td>
<td>22/53</td>
<td>ND</td>
<td>5/53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(49)</td>
<td>(41.5)</td>
<td></td>
<td>(9.5)</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>32/78</td>
<td>40/78</td>
<td>ND</td>
<td>6/78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(41)</td>
<td>(51)</td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>D42H B/W no. 1</td>
<td>B220</td>
<td>30/81</td>
<td>40/81</td>
<td>0</td>
<td>11/81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(37.5)</td>
<td>(49)</td>
<td></td>
<td>(13.5)</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>41/96</td>
<td>40/96</td>
<td>0</td>
<td>15/96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(42.7)</td>
<td>(41.6)</td>
<td></td>
<td>(15.6)</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>31/73</td>
<td>32/73</td>
<td>2/73</td>
<td>8/73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(42.4)</td>
<td>(43.8)</td>
<td>(2.7)</td>
<td>(10.9)</td>
</tr>
<tr>
<td>D42H B/W no. 2</td>
<td>B220</td>
<td>50/90</td>
<td>23/90</td>
<td>2/90</td>
<td>15/90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(55)</td>
<td>(25)</td>
<td></td>
<td>(16.5)</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>54/93</td>
<td>13/93</td>
<td>0/93</td>
<td>26/93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(58)</td>
<td>(14)</td>
<td></td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>34/85</td>
<td>33/85</td>
<td>1/85</td>
<td>16/85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(40)</td>
<td>(39)</td>
<td></td>
<td>(19)</td>
</tr>
<tr>
<td>D42H B/W no. 3</td>
<td>B220</td>
<td>44/92</td>
<td>11/92</td>
<td>ND</td>
<td>37/92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(48)</td>
<td>(12)</td>
<td></td>
<td>(40)</td>
</tr>
</tbody>
</table>
crease the proportions of allelically included B cells (to 5–10%), because the choice of which Igk allele undergoes rearrangement during receptor editing may occur stochastically (28). Previous work (15) and the present results indeed suggest that the proportion of allelically included B cells may increase substantially in mice that are engaged in extensive receptor editing. We have shown previously that the mechanism of L chain receptor editing is not impaired in autoimmune female NZB/NZW mice compared with normal mice (22). Moreover, the censorship of autoreactive B cells in the bone marrow (central tolerance), where editing mostly or exclusively occurs, is almost as effective in nondiseased and diseased autoimmune mice (29). This finding may explain the virtual identity in the extent of L chain allelic inclusion that we observe in mature B cells between normal and lupus-prone NZB/NZW mice in the present experiments. Alternatively, allelically included B cells may be deleted in the bone marrow to the same extent as excluded cells and thus are prevented from reaching a mature phenotype in the periphery. The question still remaining is whether the few autoreactive B cells that escape this tight regulation by central tolerance and reach the periphery do so by lowering their effective BCR density via allelic inclusion. This turns out not to be the case, as a very small percentage of the allelically included mature B cells in the periphery were autoreactive (ANA or DNA positive). Conversely, a very small percentage of the autoreactive anti-DNA B cells in diseased NZB/NZW mice were allelically included (Ref. 18 and Table II). We must, therefore, assume that in most allelically included, edited B cells, both the autoreactive and the nonautoreactive (germline or nonproductive) alleles undergo further rearrangement.

Our results are in agreement with earlier studies (11, 13, 15) with regard to the close association between L chain allelic inclusion and receptor editing. The earlier discrepancy concerning the fraction of included hybridomas (18) was probably due to the difference between spontaneous and LPS-induced hybridomas. This difference suggests that included B cells are generally not activated and are not recruited to the GC for class switching and somatic mutation, possibly because of their low effective BCR density (9, 11). Our results differ, however, from those of Casellas et al. (15), who reported that >30% of hCk/mCk B cells from normal mice carried DNA and/or ANA self-reactivity conferred by either the human or the mouse L chain. Moreover, we have found little evidence for that autoreactivity in L chain–included B cells, even in diseased, lupus-prone NZB/NZW mice, which have very high titers of ANA-positive autoantibodies in their serum. In the few ANA-positive, included B cells (Table III) and hybridomas (data not shown), either the human or the mouse allele accounted for the autoreactivity. However, in such rare occurrences of ANA-positive, included cells or hybridomas (two to three cells in each category), it would be unrealistic to expect that the expression of both κ alleles would result in autoreactivity. Therefore, this result alone cannot support, in our opinion, a role for allelic inclusion in B cell tolerance.

It should be pointed out that there can be no ambiguity concerning positive and negative ANA results in the current study, as...
our laboratory has 40 y experience in clinical ANA tests on a daily basis. Although ANA does not include all the autoantibodies in lupus, it is the most representative laboratory test for human and mouse SLE, and it comprises several nuclear Ags, including dsDNA. ANA is present in essentially all human lupus sera (96.5% sensitivity; 45% specificity), whereas anti-dsDNA Ab is less prevalent but more specific for SLE (57% sensitivity; 96% specificity; Ref. 30). Furthermore, the expression of isolated pairs of H and L chains derived from included cells has the advantage that the cloned L chains are produced in similar concentrations by the transfected cells; this is not always the case in hybridomas, in which the two included L chains are often produced in unequal amounts.

The ANA results make it highly unlikely that under disease conditions—that is, under intrinsic or extrinsic loss of B cell tolerance—included B cells would be activated and selected for the production of pathogenic autoantibodies (11, 15). The relative abundance of allelically included B cells in the MZ compartment supports this conclusion, because B cells in this population mostly participate in T-independent immune responses and are generally not recruited to the GC for class-switched, memory, and plasma cell formation (31, 32). The commitment of nonautoimmune, allelically included B cells to the MZ also corresponds to the signal-strength model for lineage commitment in peripheral B cells (33).

It is interesting that although κ allelic inclusion could reach a significant fraction (5–10%) of single B cells and LPS-induced hybridomas, in practice, we have not encountered Igκ/Igλ isotypic inclusion in diseased NZB/NZW mice. Although this finding is consistent with the lower efficiency of λ compared with κ rearrangement, it contrasts with certain H chain transgenic mice expressing specific autoantibodies (9, 13), in which allelic inclusion involved both κ and λ L chains. This difference may be

**FIGURE 3.** Pathways of autoreactive B cells following receptor editing and L chain exclusion or inclusion. Editing may lead to allelic exclusion or inclusion, but only L chain–included B cells that retain an autoreactive BCR use this mechanism for maintaining self-tolerance (an asterisk represents an autoreactive binding site).
explained first, by the relatively low expression of λ chains in NZB/NZW mice (18), and second, by the strong selection of λ-expressing, transgenic B cells having a particularly high affinity for their cognate Ags. This observation is well documented, for example, for the 3H9 anti-DNA autoantibody and its mutated derivatives, which bind dsDNA with a relatively high affinity (34). This example may represent a special case, in which receptor editing was induced following rearrangement as a result of autoreactivity (9); however, this process could not eliminate DNA binding because λ genes are incapable of secondary rearrangements (17). The final outcome of this special situation is the survival of isotypically included B cells that retain their autoreactivity, similarly to autoreactive transgenic B cells in which the Vk gene is rearranged to Jk5, and, therefore, receptor editing is inhibited (23) and the cells resort to clonal anergy (21). However, in the majority of allelically included B cells employing the two κ alleles, no restriction on editing both alleles should exist and, therefore, most of these B cells would lose their autoreactivity. The extent of L chain isotypic inclusion in human B cells was also found to be very low (35). Despite the reported predominant autoantibody production by early immature human B cells (55–75% of all Abs; Ref. 36), it was estimated that only 0.2–0.5% of peripheral blood B cells from healthy adults express both κ and λ L chains on their cell surface (35).

While this manuscript was reviewed for publication, a research work very similar to our investigation on dual-reactive B cells in autoimmune mice has appeared in the literature. Fournier et al. (37) have constructed hCκ/mCκ-expressing MRL/lpr mice and used flow cytometry and hybridoma analysis to evaluate the role of L chain–included B cells in the autoimmune response in these mice. Their experiments led them to conclude that, in autoimmune mice, dual-κ B cells are generated at higher numbers than in healthy mouse controls, they are self-reactive more frequently than single-κ B cells, and they are highly enriched in the Ag-selected B cell subsets. None of these three conclusions can be supported by our experiments in the NZB/NZW mouse, suggesting that certain autoimmune and tolerance mechanisms may be fundamentally different in these two lupus-prone strains.

As stated in the Results, FACS analysis of included B cells from diseased hCκ/mCκ NZB/NZW mice was very similar to that of normal mice (Fig. 1A, 1E). Moreover, a large proportion of the NZB/NZW κ-included cell population, shown by FACS, has turned out to be allelically excluded by single-cell analysis. This allelic exclusion was not tested in the MRL/lpr dual-κ B cells (37). In MRL/lpr mice, allelic inclusion was not dependent on increased L chain receptor editing, as is the case in normal mice (11, 13, 15) and in NZB/NZW mice (this work); on the contrary, it was suggested (37) that the increased kinetics of dual-κ immature B cell

Table III. Properties of ANA-positive cloned Abs from NZB/NZW single B cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>L Chain Inclusion</th>
<th>H Chain</th>
<th>L Chain Inclusion H Chain</th>
<th>L Chain Inclusion H Chain</th>
<th>L Chain Inclusion H Chain</th>
<th>ANA Pattern</th>
<th>ANA Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VH + mCκ</td>
<td>VH + hCκ</td>
</tr>
<tr>
<td>H12</td>
<td>Included</td>
<td>Vhsn7.93.a4/Jγ4</td>
<td>Cv1/Jκ5</td>
<td>19-14/Jκ2</td>
<td>Homogeneous</td>
<td>Nucleolar</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Included</td>
<td>J558.47/Jγ2</td>
<td>Gj38c/Jκ1</td>
<td>Cr1/Jκ5</td>
<td></td>
<td>Nucleolar</td>
<td></td>
</tr>
<tr>
<td>9G</td>
<td>Excluded</td>
<td>J558.33/Jγ3</td>
<td>Ae4/Jκ2</td>
<td>Ce9/Jκ1</td>
<td></td>
<td>Nucleolar</td>
<td></td>
</tr>
<tr>
<td>12G</td>
<td>Excluded</td>
<td>Q52.13.40/Jγ4</td>
<td>Ae4/Jκ2</td>
<td>Ce9/Jκ1</td>
<td></td>
<td>Nucleolar</td>
<td></td>
</tr>
<tr>
<td>17G</td>
<td>Excluded</td>
<td>J558.33/Jγ2</td>
<td>Ae4/Jκ2</td>
<td>Ce9/Jκ1</td>
<td></td>
<td>Nucleolar</td>
<td></td>
</tr>
<tr>
<td>D42H control</td>
<td>Excluded</td>
<td>S107.3.62/Jκ1</td>
<td>RF/Jκ5</td>
<td>Homogeneous</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
development is a result of the reduced efficiency of receptor editing that had been previously documented in MRL/lpr mice (38, 39), but not in NZB/NZW mice (22). The frequency of allotypically included B cells in MRL/lpr mice was reportedly increased from 3–10% in the naive mature B cell population (which is similar to the frequency of this population in nonautoimmune and NZB/NZW mice) to 20–50% (5- to 10-fold) in the plasma-cell and memory B cell subsets (37). Although we have not tested these two cell populations directly, this dramatic increase in κ allotypic inclusion is not reflected in the NZB/NZW GC cell population or in spontaneous hybridomas, which represent the acti-

vated memory B cells and plasma cells (20, 24, 25). On the contrary, the number of allotypically included B cells in our experiments was drastically decreased rather than increased in spontaneous hybridomas from nonautoimmune and NZB/NZW mice, as compared with LPS hybridomas that are derived from nonactivated B cells. Some of these differences between the two autoimmune mouse models could be attributed to the aberrant Fas signaling in MRL/lpr mice, because half of the memory B cells were maintained owing to the lpr mutation (37).

Concerning autoreactivity of excluded versus included B cells and hybridomas, we have observed some nonnuclear background binding with ~50% of purified Abs derived from NZB/NZW single cells and with ~25% of hybridoma supernatants that were obtained from these mice. The same extent of nonspecific binding was observed with Abs obtained from included and excluded B cells and with hybridomas obtained from (B6 x BALB/c) F1 and NZB/NZW mice. It must be emphasized, however, that only A, and not a general H-2 reactivity (15, 37), serves as a reliable and accepted criterion for systemic lupus in the current and all previous classifications (30). This distinction is important, because strong ANA reactivity in the serum invariably distinguishes between nonautoimmune and lupus-prone mice and humans. Moreover, polyreactive Abs may stain H-2 cells nonspecifically, and this occurs equally in normal and autoimmune mice. It may well explain the unusually high proportion of autoreactive LPS hybridomas from nonautoimmune mice that were reported by Bournier et al. (37) and also by Casellas et al. (15), a situation presumably not reflected in the serum reactivity of these mice.

Acknowledgments
We thank Drs. Esther Witsch and Anne Davidson for guidance and for protocols of single B cell analysis. Drs. Hedda Wardemann and Christian Busse for teaching the technique of Ab cloning and expression from single B cells, Dr. Norman Grover for the choice and performance of statistical analyses, and Dr. Reuven Laskov for critical reading of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References

