Activation of Type B T Cells after Protein Immunization Reveals Novel Pathways of In Vivo Presentation of Peptides

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Type B T cells recognize a peptide-MHC conformer generated in recycling endosomes and eliminated by H2-DM in late endosomes; as a result, they recognize exogenous peptide, but fail to respond to the identical epitope generated from the native protein. To investigate the behavior of these cells in vivo, we generated mice transgenic for a type B TCR recognizing the 48-62 epitope of hen egg white lysozyme (HEL) presented by I-A\(^{A}\). Type B T cells responded only to peptide ex vivo, but responded in vivo to immunization with either protein or peptide in the presence of Freund’s adjuvant or LPS. Presentation of the type B conformer was MyD88-independent, evident within 24 h after HEL immunization, and restricted to the CD11b/c\(^+\) APC subset. Immunization with listeriolysin O, a potent inducer of cell death, also primed type B T cells in vivo, and transfer of HEL-bearing allogeneic dendritic cells activated type B T cells. We conclude that a number of conditions in vivo, some of which induce inflammation and cell death, lead to peptide presentation through mechanisms distinct from the classical pathways involving H-2DM molecules. The Journal of Immunology, 2007, 178: 122–133.

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Materials and Methods

Mice

B10.BR mice were purchased from The Jackson Laboratory. 3A9 TCR-transgenic mice were a gift from Dr. M. Davis (Stanford University, Stanford, CA). Mice expressing a membrane-linked form of HEL (mHEL) under the class II promoter were previously described (4).

To generate MLA11.2 TCR-transgenic mice, genomic DNA from the MLA11.2 hybridoma (15) was amplified by PCR and cloned into the pCR2.1 vector (Invitrogen Life Technologies), then subcloned into the shuttle vectors pTαcass and pTβcass (provided by Dr. D. Mathis, Joslin Diabetes Center, Boston, MA; Ref. 16). Shuttle vectors containing the MLA11.2 α- and β-chains were linearized, gel purified by electroelution, and desalted using Elutip columns (Schleicher & Schuell Bioscience), then microinjected into oocytes from C57BL/6 mice, and fertilized oocytes were implanted into pseudopregnant females. Founders were screened by flow cytometry for expression of the transgenic TCR using the MLA11.2 TCR; 37% of CD4+ lymphocytes from transgenic mice are Vα3.2+ (left), compared with 1.8% in negative littersmates (center); typical background frequency is 1–2%. The 1G12 anti-clonotype mAb was used to detect the 3A9 TCR; 58% of CD4+ lymphocytes from 3A9 mice are 1G12+ (right). Background detection by this Ab is negligible (data not shown).

Antibodies

Anti-CD4 Ab was purchased from eBioscience; anti-V(8.8)1/8.2 and anti-Vα3.2 Abs were purchased from BD Biosciences. The 1G12 Ab, which is specific for 3A9 TCRs, has been previously described (4). Allophycocyanin-conjugated streptavidin was purchased from Molecular Probes.

Bacteria

Listeria monocytogenes strain EGD was used for all experiments; bacteria were stored as frozen glycerol stocks and thawed once before dilution into pyrogen-free saline. Mice were infected with 10⁶ organisms via i.p. injection in a total volume of 500 μl.

Generation and purification of LLO

Plasmid vectors expressing His-tagged LLO were provided by Dr. D. Portnoy (University of California, Berkeley, CA). LLO was purified as described previously (17) using Ni-NTA-Sepharose (Qiagen) and dia lyzed extensively against storage buffer (50 mM phosphate/acetate, 1 M NaCl, 1 mM EDTA, and 5 mM DTT (pH 6.0)); purity was confirmed by SDS-PAGE. Aliquots were stored at −80°C and thawed immediately before use.

Immunizations

HEL was purchased from Sigma-Aldrich and further purified in our laboratory to remove contaminants, including LPS; all HEL used for immunization was verified as containing <0.1 EU/mg. 48–62 peptide was synthesized in our laboratory as described previously (17) using Ni-NTA-Sepharose (Qiagen) and dialyzed extensively against storage buffer (50 mM phosphate/acetate, 1 M NaCl, 1 mM EDTA, and 5 mM DTT (pH 6.0)); purity was confirmed by SDS-PAGE. Aliquots were stored at −80°C and thawed immediately before use.

CFSE labeling and adoptive transfer

Cells were labeled with CFSE using the Vybrant cell-labeling kit (Molecular Probes). T cells for labeling were isolated from the spleens and inguinal, mesenteric, cervical, and brachial lymph nodes of transgenic mice; single-cell suspensions were generated by disruption with the back of a sterile syringe and passage through a 70-μm Falcon cell strainer (BD Biosciences). Erythrocytes were lysed using ammonium chloride and cells
were washed in PBS containing 0.1% BSA (Sigma-Aldrich). Lyophilized CFSE was resuspended in DMSO to a concentration of 5 mM, then diluted 1/3000 in PBS prewarmed to 37°C (i.e., final labeling concentration of 1.67 \mu M). Cells were resuspended in PBS plus CFSE at a concentration of 10^7/ml; the labeling reaction was allowed to proceed for 20 min at 37°C, then stopped by addition of an equal volume of DMEM plus 5% FCS. Before transfer, cells were washed twice more in PBS plus 0.1% BSA, then resuspended in the appropriate volume of ice-cold PBS. Labeled cells were

FIGURE 2. Characterization of MLA11.2 TCR-transgenic mice: flow cytometric analysis of primary and secondary lymphoid organs. A, Spleens (left column), lymph nodes (center), and thymi (right) were harvested from MLA11.2, negative littermate, and 3A9 mice sacrificed at 6 wk of age. Single-cell suspensions were generated and cells were stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. B, Lymph node cells from MLA11.2 and 3A9 mice (the same samples analyzed in A) were gated on CD4 single-positive lymphocytes and further analyzed for expression of CD69 (left) and CD62L (right). CD4^+ lymphocytes from both transgenic mice are CD69^{low} and CD62L^{high}, indicating retention of a naive phenotype. Data shown are representative of three independent experiments which yielded similar results.
injected i.v. via the tail vein with a total volume of 0.5 ml; mice typically received $5 \times 10^7$ cells, of which 5–10% were CD4$^+$ T cells expressing the transgenic TCR. In some cases, cells were injected via the retro-orbital vein rather than the tail vein; this did not affect recovery or proliferation of the cells.

In all experiments, mice receiving labeled T cells were immunized/infected 24 h posttransfer; 72 h after immunization (i.e., 96 h posttransfer), mice were sacrificed and draining lymph nodes (popliteal for footpad immunizations, mesenteric for i.p. injections) were harvested. Single-cell suspensions were generated as described above; cells were washed in FACS buffer (PBS plus 2% FCS plus 0.02% sodium azide), stained with anti-CD4-PE and anti-V$\alpha$3.2-biotin (1G12-biotin for 3A9 T cells) followed by streptavidin-allophycocyanin, and analyzed for CFSE dilution by flow cytometry. Data were analyzed using FlowJo version 6.3.2 (Tree Star Software). Quantitation of data was performed using the proliferation analysis function of FlowJo; all cells which had undergone at least one division were included in calculation of the average number of divisions per cell.

Cell separation and T cell assays

APC subsets (CD11b/c$^+$ and CD19$^+$) were isolated using magnetic bead sorting (Miltenyi Biotec). Popliteal lymph nodes were harvested, digested with collagenase, and pressed through a 70-µm cell strainer; then incubated simultaneously with anti-CD11c and anti-CD11b beads at 4°C for 20 min. Labeled cells were positively selected on two successive columns to maximize purity and the pooled flow-through was passed through an additional negative selection column to remove any residual labeled cells. This procedure was then repeated using anti-CD19 beads to isolate the CD19$^+$ subset. Purity was checked by flow cytometry using anti-CD11b, anti-CD11c, and anti-B220 Abs (BD Biosciences). In all experiments, the CD11b/c$^+$ subset was $>70\%$ pure, with most contaminating cells expressing B220, while the CD19$^+$ subset was $>96\%$ pure (data not shown).

To test proliferation of primary T cells, spleens and lymph nodes were harvested from transgenic mice and single-cell suspensions were generated by passage through a 70-µm cell strainer; cells were resuspended in DMEM plus 10% FCS and plated at $5 \times 10^5$ cells/well in 96-well round-bottom tissue culture plates. Titrating doses of HEL were added and plates were incubated at 37°C in 5% CO$_2$. Proliferation was measured by [H]thymidine incorporation over the last 24 h of a 96-h culture.

To test proliferation of T cell hybridomas, APCs (e.g., isolated from draining lymph nodes of immunized mice) were resuspended in medium and plated at titrating numbers in 96-well round-bottom tissue culture
are representative of three independent experiments.

plates. T hybridoma cells (1 × 10^5/well) were added in a total volume of 200 μl, and plates were incubated at 37°C in 5% CO₂ for 18 h. A total of 100 μl of supernatant was then removed, repeatedly frozen and thawed to lyse residual cells, and tested for its ability to stimulate proliferation of the IL-2-dependent indicator line CTLL-2, measured by [3H]thymidine incorporation over the last 8 h of a 24-h culture.

**Results**

**Generation and characterization of type B TCR-transgenic mice**

To generate a transgenic mouse, we scanned our existing bank of type B T cell hybridomas to select a TCR with a single in-frame α- and β-chain, and, to facilitate screening, available mAbs to them. The MLA11.2 hybridoma fit both these criteria, as it expressed a single in-frame α- (Vα3.2) and β-chain (Vβ8), the same as 3A9, the type A T cell to which it is compared in these studies (15). The TCR α- and β-chain genes were amplified by PCR, cloned into the shuttle vectors of Benoist and Mathis and colleagues (Ref. 16 and Fig. 1A) and microinjected into oocytes; eight founders that integrated the transgene were identified (representative shown in Fig. 1B). Flow cytometric analyses of PBLs for the MLA11.2, negative littermate, and 3A9 (type A TCR-transgenic) mice are shown in Fig. 1C; in nontransgenic mice (i.e., negative littermate), ~2% of CD4^+ T cells used Vα3.2 (Fig. 1C, center panel), while in MLA11.2-transgenic mice, this frequency increased to ~30% (Fig. 1C, left panel).

MLA11.2-transgenic mice (Fig. 2A, top row) did not differ significantly from negative littermates (Fig. 2A, middle) in their distribution of lymphocyte and thymocyte subsets (Fig. 2A). We further analyzed peripheral CD4^+ T cells to ascertain their activation state; MLA11.2-transgenic T cells were CD69low (Fig. 2B, left) and CD62L^{high}, indicating that they retained a naive phenotype in the periphery (Fig. 2B). In sum, primary MLA11.2-transgenic T cells appeared to be normal, both in their ability to navigate thymic selection and in their peripheral phenotype.

Of note, the prevalence of transgenic T cells in MLA11.2-transgenic mice was less than that found in 3A9-transgenic mice, in which >60% of peripheral CD4^+ T cells expressed the transgenic TCR (Fig. 1C, right panel) and the percentage of CD4 single-positive cells in both the thymus and periphery was increased relative to nontransgenic controls. This discrepancy likely reflects poor thymic selection of the MLA11.2 TCR relative to the 3A9 TCR. To circumvent this issue, MLA11.2 TCR-transgenic mice were crossed to mice lacking the constant region of the TCRα locus (α/⁻/⁻) to prevent endogenous rearrangement. Lymphocytes from MLA11.2 mice on the α/⁻/⁻ background displayed uniform staining for Vα3.2, with >99% of CD4^+ T cells expressing the transgenic TCR; these results were similar to those seen in 3A9-transgenic mice, in which nearly all CD4^+ T cells stained with the 1G12 clonotypic Ab (Fig. 3A). Furthermore, both transgenic T cells were similar in their selection bias; Vα3.2^+ cells from MLA11.2-transgenic mice and 1G12^+ cells from 3A9-transgenic mice skewed equally toward the CD4^+ lineage, whether taken from spleens (Fig. 3B) or lymph nodes of the transgenic mice (Fig. 3C).

**Primary type B T cells recapitulate the hybridoma phenotype ex vivo**

We tested the ability of T cells from the transgenic mice to proliferate ex vivo in response to HEL and to 48–62 peptide. As shown in Fig. 4, cells from either the spleen or lymph nodes of MLA11.2 mice proliferated specifically, and with relatively high sensitivity (half-maximal stimulation at ~0.1 μM), in response to 48–62 peptide; however, they responded weakly to HEL, with
proliferation observed only at the highest dose tested (30 μM) and only in splenocytes (Fig. 4, left panels). In contrast, cells from nontransgenic littermates did not proliferate in response either to peptide or to HEL, while cells from 3A9-transgenic mice proliferated in response to both (Fig. 4, center and right panels). Similar results were obtained when assessing IL-2 production by CTLL assay (data not shown). Thus, primary type B T cells precisely recapitulated the hybridoma phenotype ex vivo.

Type B T cells respond in vivo to HEL in the presence of CFA or LPS

To investigate priming of type B T cells in vivo, cells from the spleens and lymph nodes of transgenic mice were labeled with CFSE and adoptively transferred into secondary hosts; following immunization of the recipient mice, the transgenic T cells were recovered from the draining lymph node and the extent of their immunization of the recipient mice, the transgenic T cells were spleens and lymph nodes of transgenic mice were labeled with Type B T cells respond in vivo to HEL in the presence of CFA or LPS

Infection with Listeria or soluble LLO induces presentation of the type B conformer

One potential mechanism by which the type B conformer might be generated in vivo is through phagocytic uptake of endogenous, Ag-bearing cells undergoing cell death. To test this hypothesis, we initially assessed priming of MLA11.2 T cells in mHEL mice, which express HEL as a membrane-linked protein under control of the Eoo promoter (4). These mice express very high levels of HEL in all class II MHC-expressing cells and present high levels of the 48-62/2-Ak complex, estimated as ~20,000 complexes per APC; by comparison, ~300 complexes per cell are sufficient for T cell activation (4). MLA11.2 T cells adoptively transferred into mHEL mice underwent one to two divisions, indicating that some basal level of priming occurred in these mice regardless of inflammation (Fig. 7A); this likely represents the in vivo correlate of the response to high doses of HEL observed ex vivo (cf Fig. 4). Administration of CFA induced responding cells to undergo an average of one additional division relative to basal proliferation with pyrogen-free saline (PFS; 3.05 vs 2.08 divisions/cell) and significantly increased the percentage of cells which underwent more than three divisions (28.5 vs 8.4%). Infection of mice with 10^9 L. monocytogenes also enhanced the response of the type B T cells, inducing proliferation similar to that seen with CFA (3.01 divisions/cell, 26.8% undergoing more than three divisions). Administration of IFA did not significantly enhance proliferation; the response of type A T cells under these conditions could not be assessed due to activation-induced death of the adoptively transferred cells in mHEL mice (data not shown).

We next investigated whether LLO alone was sufficient to drive priming of type B T cells in mHEL mice. LLO, a major virulence factor of L. monocytogenes, is a member of the cholesterol-dependant cytolysin family and a potent inducer of both necrotic and apoptotic cell death (18, 19), and injection of LLO in the hind footpad induces cell death in the draining lymph node (19). Injection of mHEL mice in the hind footpad with 120 pM LLO (an apoptogenic, but sublytic dose; Ref. 19) resulted in significantly greater proliferation of adoptively transferred MLA11.2 T cells than that observed in the PBS control (Fig. 7B); enhancement of proliferation with LLO was significantly greater than that seen with live Listeria (cf Fig. 7B with Fig. 7A). This effect was not limited to endogenously expressed HEL, as coadministration of B10.BR mice with HEL and LLO induced proliferation of MLA11.2 T cells, while injection of HEL alone induced only a minimal response. In contrast, administration of HEL alone was sufficient to prime 3A9 T cells and coadministration of LLO had little, if any, effect (Fig. 7C). Ag-independent priming (i.e., in response to LPS alone) was not observed in any experiment. Thus, in contrast to type A T cells, priming of type B T cells was completely dependent on the presence of adjuvant, indicating that an inflammatory response was required.

In a previous study, we determined that unlike the type A conformer, which persists for several weeks at 37°C, the type B conformer quickly loses its ability to stimulate T cells ex vivo (3). To assess the kinetics of in vivo generation of the type B conformer, we measured proliferation of CFSE-labeled MLA11.2 T cells while varying the interval between immunization and T cell transfer. When mice were immunized with HEL and LPS 72 h before transfer, proliferation of the cells was significantly reduced compared with when mice were immunized 24 h after T cell transfer, as in our previous experiments (Fig. 6). By contrast, proliferation of the type A T cell 3A9 was unchanged when mice were immunized 72 h before transfer as opposed to 24 h after transfer (see Fig. 6 legend and Table I). Thus, the type B conformer generated in vivo is short-lived; this corroborates our results with ex vivo generation of the type B conformer.

Generation of the type B conformer is MyD88 independent and can occur through uptake of cell-associated Ag

The adjuvant effect of LPS is mediated through stimulation of TLR4 on APCs, which activates two downstream signaling pathways, one requiring the adaptor molecule MyD88 and another independent of this molecule (reviewed in Ref. 20). To determine whether LPS-induced priming of type B T cells was MyD88 dependent, proliferation of CFSE-labeled MLA11.2 T cells in MyD88−/− mice in response to HEL/LPS immunization was examined. Priming of the type B T cells in MyD88−/− mice was indistinguishable from that observed in wild-type controls (Fig.
Table I. Quantitation of cell proliferation for CFSE dilution experiments

<table>
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<tr>
<th>T Cell</th>
<th>Immunogen</th>
<th>% Divided</th>
<th>Average No. of Divisions</th>
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</thead>
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</tr>
<tr>
<td>0.1 nM HEL/CFA&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1.19</td>
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<tr>
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<td>1.50</td>
<td></td>
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<tr>
<td>1 nM 48–62/CFA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.4</td>
<td>1.61</td>
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<tr>
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<tr>
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<td></td>
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<td></td>
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<td>2.31</td>
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<tr>
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<td>56.5</td>
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<sup>a</sup>Data were quantitated using FlowJo software as described in Materials and Methods; all cells that had undergone at least one division were included in calculation of the average number of divisions.

<sup>b</sup>cf Fig. 5.

<sup>c</sup>cf Fig. 6.

<sup>d</sup>cf Fig. 7.

<sup>e</sup>cf Fig. 8. Quantitation for Fig. 7, A and B (transfer into mHEL mice), is included along with the relevant text. DCs, Dendritic cells.

FIGURE 5. Proliferation of adoptively transferred MLA11.2 T cells in vivo. A, MLA11.2-transgenic T cells were labeled with CFSE and transferred i.v. into B10.BR mice. Twenty-four hours later, mice were immunized in the hind footpad with CFA alone (left) or with 10 nM HEL (center) or 48–62 (right) in CFA. Mice were sacrificed and popliteal lymph nodes harvested and analyzed for CFSE dilution 72 h postimmunization. Data are representative of three independent experiments. B, MLA11.2 T cells were labeled with CFSE and transferred into B10.BR mice as above and mice were immunized in the hind footpad with 0.1 nM (left), 1 nM (center), or 10 nM (right) of either HEL (red) or peptide (blue) in CFA. Analysis of CFSE dilution was performed as in A. Data are representative of two independent experiments. C, MLA11.2 T cells were labeled with CFSE and transferred into B10.BR mice as above and mice were immunized in the hind footpad with 10 µg of E. coli LPS, 10 nM HEL in PFS, or 10 nM HEL coinjected with the indicated dose of LPS. Analysis of CFSE dilution was performed as in A. Proliferation of CFSE-labeled 3A9 T cells was also assessed in response to immunization with 10 nM HEL in the absence or presence of 10 µg of LPS (bottom right panel). Data are representative of four independent experiments.
lymph node 24 h postimmunization, and CD11b/c\textsuperscript{+} and CD19\textsuperscript{+} APCs were isolated using magnetic beads and tested for their ability to stimulate type A and B hybridomas. As in our previous study, we found that the type A conformer was presented by CD11b/c\textsuperscript{+} cells (Fig. 9A), as well as by CD19\textsuperscript{+} B cells (Fig. 9B). Co-administration of LPS enhanced presentation by 30- to 100-fold in both cell types. Further separation of CD11b\textsuperscript{+} from CD11c\textsuperscript{+} cells did not indicate significant differences in presentation between these cells (data not shown). The type B conformer was also presented by CD11b/c\textsuperscript{+} cells 24 h after immunization with HEL and LPS, although larger numbers of APCs were required for stimulation (Fig. 9C). Presentation was LPS dependent, consistent with the results of our adoptive transfer studies. Presentation was best noted with a second type B T cell, CP1.7, which is 10- to 30-fold...
more sensitive than MLA11.2 (15). In contrast to the type A conformer, we did not observe any presentation of the type B conformer by CD19/H11001 B cells (data not shown), indicating that either the type B conformer was presented by B cells at too low a level to stimulate the type B hybridoma, or that its presentation in vivo was restricted to macrophages and/or dendritic cells. In a second protocol, we recovered cells from the draining lymph node 24 h after immunization with HEL in CFA and tested the response of MLA11.2 to either CD11c/H11001 or CD11b/H11001 cells. We observed a small degree of presentation by the CD11b/H11001 subset, which was not seen with CD11c/H11001 cells (Fig. 9D). In sum, APCs recovered from immunized lymph nodes 24 h after immunization with HEL contain type B complexes, albeit in relatively small amounts compared with the type A complex.

**FIGURE 8.** Generation of the type B conformer in vivo is MyD88 independent and can occur by uptake of cell-associated Ag. A, MLA11.2-transgenic T cells were labeled with CFSE and transferred i.v. into B10.BR mice (left) or MyD88−/− mice backcrossed onto the B10.BR background (right). Twenty-four hours later, mice were immunized in the hind footpad with 10 nM HEL and 1 μg of LPS. Mice were sacrificed and popliteal lymph nodes harvested and analyzed for CFSE dilution 72 h postimmunization. B, MLA11.2 T cells were labeled with CFSE and transferred into B10.BR mice as above. Twenty-four hours later, mice were immunized in the hind footpad with CD11b/H11001 cells isolated from C57BL/6 (H-2b) mice (left) or B10.BR mice (right) which had been pulsed for 2 h with 10 μM HEL in the presence (red) or absence (blue) of 1 μg/ml LPS. Analysis of CFSE dilution was performed as in A. Data are representative of two independent experiments.

**FIGURE 9.** APCs in the draining lymph node present the type B conformer following immunization with HEL in adjuvant. A–C, B10.BR mice were injected in the hind footpad with 10 nM HEL (open symbols) or 10 nM HEL plus 1 μg of LPS (filled symbols). Twenty-four hours postimmunization, mice were sacrificed and draining lymph nodes were harvested. CD11b/H11001 and CD19/H11001 cells were purified using magnetic beads and plated at limiting numbers in 96-well round-bottom plates, and cells of the 3A9 (type A) or CP1.7 (type B) T cell hybridomas were added at 5 × 10^4 cells/well. After incubation overnight at 37°C, supernatants were tested for IL-2 content as measured by CTLL assay. D, B10.BR mice were injected in the hind footpad with 10 nM HEL in CFA. Draining lymph nodes were harvested 24 h after immunization and CD11c/H11001 (triangles) and CD11b/H11001 11c− cells (squares) were isolated using magnetic beads and plated at limiting numbers in 96-well round-bottom plates. MLA11.2 hybridoma cells were then added at 5 × 10^4 cells/well and their response tested as in A–C. In all graphs, each data point represents the mean cpm of triplicate wells; error bars represent the SD.
Discussion

The studies reported here establish conditions that lead to activation of type B T cells in response to protein Ags in vivo. Presentation of the type B conformer did not occur in response to immunization with HEL alone, but was observed with HEL immunization in the presence of stimuli that induced inflammation, including CFA, LPS, and LLO. Priming was also observed in response to endogenous HEL expressed at high levels in APC, and, finally, transfer of allogeneic or syngeneic APC carrying HEL induced activation of type B T cells. Taken together, these findings indicate that pathways exist in vivo that result in presentation of the type B conformer from processing of intact protein Ags.

Presentation of the type B complex indicates that a peptide binds to a class II MHC molecule at a site within the APC devoid of DM molecules. Presentation of this conformer following processing of an intact protein (e.g., HEL) could indicate a unique mode of intracellular generation of the peptide-MHC complex; the peptide may be released from the processing vesicles of the APC into the extracellular milieu, subsequently loading in trans as an exogenous peptide, or intracellular trafficking of peptides by the activated APC may be altered, such that peptide-MHC complex formation occurs at another cellular site where DM is absent, such as in early endocytic vesicles. Alternatively, presentation of the type B conformer could reflect a unique mode of uptake of the Ag by the APC, such as through phagocytic uptake of Ag-bearing cells which have died or are in the process of undergoing apoptosis. Of note, presentation of cell-associated Ags on class II MHC is quite robust, with studies estimating its efficiency to be 100- to 1000-fold greater than for soluble Ag (23, 24). Our results indicate that peptides derived from HEL must have been presented in vivo through pathways in which DM did not participate, i.e., in pathways distinct from the classical one in which the protein is processed and complexes assembled in DM-bearing vesicles involving MHC molecules recently arrived from the endoplasmic reticulum-Golgi (reviewed in Ref. 25).

Two inflammatory stimuli that directly or indirectly activated APCs, CFA and LPS, resulted in presentation of the type B complex. The lack of a requirement for the adaptor molecule MyD88 suggests that activation of this unique pathway of presentation was mediated by MyD88-independent TLR signaling, i.e., through activation of TRAM/Trif (26, 27). However, we have no clear indication of the route of presentation and the precise mechanism responsible for generation of the type B conformer and on pathways under investigation. To date, we have been unable to reproduce the conditions required for generation of the type B conformer from intact HEL ex vivo; treatment of bone marrow-derived dendritic cells with LPS induced neither presentation of HEL to type B hybridomas nor release of HEL fragments. The reasons for these results are unclear, but likely indicate that in vivo, there are interactions between multiple cell types not found in ex vivo culture. For example, inflammation induced by these two stimuli might have resulted in death of some of the HEL-bearing cells, with ensuing uptake and presentation in trans by APCs. Certainly, LPS can affect cell viability, both as a result of necrotic death secondary to NO production and to the oxidative burst and from apoptosis of B cells mediated by LPS-induced up-regulation of Fas ligand (28).

The fact that presentation was induced by immunization with LLO strongly suggests a pathway involving uptake of Ag present on dead and dying cells. LLO is strongly apoptogenic both in vitro and in vivo; while LLO can also induce necrotic death via perforation of cell membranes, the doses of LLO used in this study were insufficient to cause this result (19). In vivo, priming of CD4+ T cell responses to L. monocytogenes is thought to occur through presentation of listerial Ags following uptake of infected cells by host macrophages and dendritic cells (29). Thus, the route of presentation of the type B conformer in vivo could well include such a mechanism if some of the cells taking up HEL die as a result of the inflammatory process. Furthermore, the fact that immunization with allogeneic APCs pulsed with HEL in vitro resulted in presentation of the type B complex indicates that intercellular transfer of the epitope can, indeed, take place; whether this transfer is the same as that taking place upon immunization with CFA, LPS, or LLO remains to be determined. Cell-associated Ags are taken up in phagolysosomal vesicles which may result from fusion of the endoplasmic reticulum with the plasma membrane, in contrast to the endocytic vesicles involved in uptake and presentation of soluble Ags (30). The properties of these vesicles with respect to Ag processing and presentation have not been studied in detail and they may well differ significantly from conventional MHC class II-loading vesicles. If DM were absent or inactive in these vesicles, or if the rate of transit through the pathway overwhelmed the capacity of DM to function as a conformational editor, then presentation of the type B conformer would be the expected result. Alternatively, phagolysosomal proteases may preferentially generate truncated peptides that lack flanking residues necessary for DM to eliminate the type B conformer; this would result in presentation of the type B conformer, as the complexes that form would be resistant to the editing function of DM (31).

Finally, in contrast to the established role for inflammation in up-regulating costimulatory molecules through stimulation of TLRs, the extent to which Ag processing and presentation themselves are affected by the presence of an inflammatory milieu remains controversial. Several groups have shown that the repertoire of peptides presented from a given protein Ag is affected by the presence of proinflammatory cytokines (32, 33); however, the relevance of this observation to the immune response is unclear, as the magnitude of the T cell response to a given epitope correlates poorly with its quantitative level of presentation (34), a finding that holds even in the absence of costimulation (35). Presentation of the type B conformer in vivo may thus represent a novel mechanism by which innate immunity can influence adaptive immune responses by affecting Ag processing and presentation. Further studies should shed light on the mechanism by which inflammation-activated APCs are induced to present the type B conformer and on the nature of the intracellular compartment in which formation of the type B conformer occurs in vivo; such knowledge will be critical in evaluating the role played by type B T cells in autoimmune and in immunity to infection.

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Disclosures

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