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Both B cells and dendritic cells (DCs) have been implicated as autoantigen-presenting cells in the activation of self-reactive T cells. However, most self-proteins are ubiquitously and/or developmentally expressed, making it difficult to determine the source and the exposure of autoantigens to APCs in a controlled manner. In this study, we have used an Ig transgenic mouse model to examine the mechanisms by which B cells and other APCs acquire and present lupus autoantigens in vivo. Targeting a lupus autoantigen, the small nuclear ribonucleoprotein particle D protein, to the BCR activates autoreactive T cells in the periphery. Our in vivo studies demonstrate that autoantigen-specific B cells, when present in the repertoire, are the first subset of APCs to capture and present self-proteins for activating T cells. Thereafter, DCs acquire self-Ag and become effective APCs for stimulating the same subsets of autoreactive T cells. This mechanism provides one explanation of how early steps in autoimmunity can focus responses, via BCR, at a small group of self-proteins among the total milieu of intracellular self-proteins. Subsequently, DCs and other professional APCs may then amplify and perpetuate the autoimmune T cell response. The Journal of Immunology, 2006, 177: 4481–4487.

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or shape the autoimmune T cell response toward specific self determinants that may then be perpetuated by other professional APCs such as dendritic and/or macrophages.

Materials and Methods

Ig transgenic mice

Ig transgenic mice expressing the Vh of an anti-NP Ab have been fully backcrossed onto BALB/c and MRL lpr/lpr backgrounds (23). To eliminate endogenous H chain expression, the Tg mice were backcrossed with BALB/c J<sub>H<sub>3</sub> knockout mice. The α<sub>1</sub> B cells (~3% of splenic B cells) in these mice recognize the hapten NP.

Anti-snRNP B cell hybridoma, designated 2-12, was originally cloned from an MRL lpr/lpr mouse (24). Ig transgenic mice were derived using the rearranged VDJ segment of the 2-12 anti-snRNP hybridoma cloned upstream in a vector containing the C<sub>μ</sub> region gene segment (25). Approximately 30% of splenic B cells maintain specificity for the mouse snRNP autoantigen as presented herein and previously (2, 25). Transgenic founders were backcrossed for >10 generations to B10.A, C57BL/6 mice or MRL lpr/lpr mice. Presence of the transgene was identified by PCR analysis of tail DNA as described previously (2). Animals were age matched in all experiments and were housed in a specific pathogen-free facility at Yale University.

Purification of recombinant snRNP D fusion protein

Recombinant murine snRNP D protein expressed in Escherichia coli was used as the stimulating Ag in T cell proliferation assays. Recombinant mouse snRNP D protein was purified by anion and cation exchange chromatography as previously described, and was absorbed for potential mites by anti-LPS column chromatography using agarose beads coated with polynynx B (Pierce) (26). In some experiments, snRNP D protein was labeled with biotin for fluorescence staining and immunoblotting.

snRNP D protein binding, B cell purification, and Ag loading

B cells were purified by negative selection using magnetic beads (Milteny Biotec). In brief, lymphocytes from spleenocytes were enriched by Ficoll-Hypaque (Sigma-Aldrich) density separation. Cells were labeled with anti-mouse CD43 microbeads and passed through a magnetic separation column. B cells were further incubated with biotinylated snRNP D protein, followed with streptavidin-beads (Milteny Biotec) and again purified on magnetic columns. The purity of snRNP D binding B cells was ~85% as assessed by flow cytometry. snRNP D protein binding B cells and non-specific B cells were loaded with biotinylated recombinant snRNP D protein in vitro by incubating cells at 2 × 10<sup>6</sup> cells with 0.08, 0.4, or 0.8 μg of biotin-snRNP D for 2 h at 37°C.

Immunoblot quantitation for snRNP D protein uptake by BCR

B cells pulsed with Ag were washed with PBS, pelleted, and lysed in immunoprecipitation assay buffer as previously described (27). Lysates were run as a 12% PAGE gel and transferred to nitrocellulose membrane. snRNP D protein uptake was detected by probing with streptavidin-conjugated horseradish peroxidase and visualized with chemiluminescence. Titrations of biotinylated snRNP D protein was used as a positive control and concentration standard in immunoblots.

Haptenated snRNP D self-Ag

A portion of the snRNP D protein was haptenated with NP-hydroxy-succinimide ester (Biosearch Technologies) as described previously for immunization or in vitro T cell recall stimulation assay (23). In these experiments, anti-NP Tg mice (BALB/c or MRL lpr/lpr) were immunized with 50 μg of NP-snRNP D protein or snRNP D protein alone with CFA in a single hind footpad. After 14 days, T cells were purified for recall proliferation assays as described above.

Purification of CD11c<sup>+</sup> DCs

Spleens from immunized anti-NP Tg mice were harvested for CD11c<sup>+</sup> DC purification. In brief, spleens were injected with collagenase D solution, and then cut into smaller pieces. The spleen fragments were further incubated for 30 min at 37°C and passed through a 70-μm cell strainer. Single cell suspensions were labeled with anti-CD11c microbeads (Miltenyi Biotec). CD11c<sup>+</sup> DCs were positively selected using an LS column. The purity of CD11c<sup>+</sup> cells was >80% as determined by flow cytometry.

T cell proliferation assays

Spleenic CD4 T cells were purified by negative selection, using anti-mouse B220, anti-mouse MHC class II, anti-mouse CD11b, and anti-mouse CD8 microbeads (Miltenyi Biotec) or positive selection, using anti-mouse CD4 microbeads. The purity of CD4<sup>+</sup> T cells was >94% as assessed by flow cytometry. Conventional T cell proliferation assays were performed with CD4<sup>+</sup> T cells (2 × 10<sup>5</sup>/well) and irradiated purified B cells (500 rad) as APCs at 5 × 10<sup>6</sup>/well. All assays were set up in triplicate and incubated with or without Ag for 3 days. Lymphocyte proliferation was assessed by [<sup>3</sup>H]thymidine incorporation (1.0 μCi/well; ICRN Chemicals) during the last 18 h of culture. Sample wells were harvested onto filters, and incorporated radioactivity was counted in a Betaplate liquid scintillation counter (LKB/Wallac).

Results

Self-Ag processed by Ag-specific B cells activates autoreactive T cells in vivo

Our previous studies have demonstrated that anti-snRNP Ig Tg B cells capture endogenous self-Ag and activate autoreactive T cells in vitro (2). However, both our own prior studies and those of others could not control for the presentation of ubiquitous endogenous self-Ag by other subsets of APCs in vivo. The use of transgenic animals with BCR specificity lacking secreted Ab (mlg Tg) allowed us to clearly interpret B cell APC function in the absence of Ag presentation via immune complexes or Fc receptors on other APCs. To directly control the presence and uptake of autoantigen by B cell APCs, NP-modified snRNP D protein was exposed to anti-NP B cells (Fig. 1A). Because the anti-NP B cells do not secrete Ab, soluble immune complexes will not be generated in this model as a potential source of autoantigen presentation by other FcR-bearing APCs.

Membrane Ig Tg BALB/c mice or wild-type mice were immunized with either NP-conjugated snRNP D or unmodified snRNP D protein. To assess initial in vivo priming, purified CD4<sup>+</sup> T cells from these animals were then exposed to anti-NP B cell APCs with or without NP-snRNP D. As shown in Fig. 1B, anti-snRNP T cells could be primed in anti-NP BALB/c transgenic mice only when immunized with NP-snRNP Ag. The lack of soluble Ig in these mice excludes any contribution of immune complexes to these autoimmune T cell responses and detectable T cell autoimmunity required autoantigen binding BCRs. In contrast, unmodified snRNP D protein, unable to be bound by anti-NP-specific B cells or B cells from wild-type mice, fails to break T cell tolerance in these animals. The autoimmune response was not related to NP modification of the autoantigen because NP-linked self-Ag failed to break autoreactive T cell tolerance in wild-type mice where BCR also fail to bind specific autoantigen (Fig. 1B). The fraction of B cells capable of binding NP Ag is ~3% of the splenic B cell repertoire that is dependent on the endogenous expression of α1 Ig L chains (23). Thus, when Ag is not directly targeted to B cells via BCR, professional APCs fail to break T cell tolerance despite their exposure to the snRNP immunogen. Finally, these data also demonstrate that snRNP autoantigene T cells exist in the repertoire of normal mice and can be triggered by autoantigen-specific B cells as APCs if they are a part of the normal repertoire. No evidence exists from our work or the studies of others that the autoimmune T cell repertoire is altered in anti-NP transgenic animals. Moreover, anti-NP B cells in transgenic mice are resting and do not express CD80 or CD86 before Ag binding (23) (data not shown). In our studies, the binding of NP-linked Ag by Tg B cells upregulated both CD80 and CD86 (data not shown).

MRL lpr/lpr mice, a murine model of human systemic lupus erythematosus, spontaneously develop anti-snRNP and anti-DNA autoantibodies as well as skin and kidney pathology. In a similar manner, anti-NP MRL lpr/lpr Tg mice could be primed for...
autoreactive T cell responses by immunization with NP-snRNP D protein (Fig. 2A). We reasoned that T cell responses provoked by NP-snRNP D protein presented by B cells should also be responsive to snRNP self-Ag acquired and presented by other, unrelated anti-snRNP transgenic B cells. Such an experiment is illustrated in Fig. 2B, where CD4⁺ T cells from NP-snRNP immune MRL lpr/lpr mice proliferate strongly in response to syngeneic anti-snRNP 2-12 Tg B cells without adding self-Ag. These data support the conclusion that snRNP-specific B cells (2-12 anti-snRNP Ig Tg B cells) have acquired and processed an endogenous source of self-Ag in activating autoreactive T cells. These studies provide additional evidence that NP-snRNP can be processed via receptor-mediated uptake in Ag-specific B cells for the subsequent priming of autoreactive T cells and this process is dependent on self-Ag. In addition, these data show that responding T are not specific for artificially altered snRNP Ag from the haptenation process because T cells are also activated by anti-snRNP Ig Tg B cells.

**FIGURE 1.** Ag-specific B cells elicit T cell autoimmunity in anti-NP membrane transgenic BALB/c mice. A, Schematic model of NP-Tg B cell Ag presentation. Anti-NP membrane Tg or wild-type BALB/c mice were immunized with NP-conjugated snRNP D protein or with unmanipulated snRNP D protein in CFA. This experimental approach examines T cell responses from mice with BCR able to bind NP-snRNP autoantigen (without secreting Ig) vs mice with nonspecific BCR. T cell recall proliferation assays were performed at day 14 after immunization in the presence of anti-NP B cells as APCs with NP-snRNP D (5 μg/ml). B, Anti-snRNP T cell proliferative responses from the experimental strategy described in A.

**FIGURE 2.** Autoreactive T cells are elicited in anti-NP MRL lpr mice and are activated by 2-12 anti-snRNP Tg B cells. Anti-NP Tg or wild-type MRL lpr/lpr were immunized with NP-conjugated snRNP D protein or with unmanipulated snRNP D protein in CFA. T cell proliferation assays were performed at day 14 after immunization in the presence of anti-NP B cells as APCs with NP-snRNP D Ag (5 μg/ml). A indicates CD4 T cell responses from immunized anti-NP MRL lpr/lpr mice stimulated with anti-NP B cells and NP-snRNP D. B indicates the CD4 T cell responses from NP-snRNP D immune MRL lpr/lpr mice (identical with those examined in A) stimulated with 2-12 anti-snRNP Tg B cells from MRL lpr/lpr mice. No snRNP D protein was added to the cultures in B.

**Autoantigen processing requires specific uptake by BCR**

In contrast to the use of anti-NP transgenic animals, ~30% of 2-12 anti-snRNP Tg B cells with the correct L chains bind snRNP Ag (25). However, this alternative transgenic system allowed us to further confirm the importance of receptor-specific autoantigen processing by B cells. We purified snRNP D binding B cells and nonbinding B cells by flow cytometry from within the same mouse repertoire (Fig. 3A). Each purified B cell population was then incubated with titrations of biotinylated snRNP D protein. B cell lysates were then immunoblotted with avidin-AP to detect the presence and receptor-mediated uptake of snRNP Ag and compared with a titration of snRNP D on the immunoblot. As shown in Fig. 3B, snRNP D binding transgenic B cells are capable of capturing even small concentrations of self-Ag. These studies demonstrate that ~8 ng of snRNP D protein is associated with the 2 × 10⁶ B cells. This process was Ag specific and could be...
with avidin-AP. Purified 2-12 Tg B cells (20, 100, and 200 ng in PAGE gel (panel A). Protein bands were probed of biotin-snRNP D were used to illustrate the density of snRNP D protein and B220-Cyc. Cells are gated on the B220 population. B, Serial dilutions of biotin-snRNP D were used to illustrate the density of snRNP D protein at 20, 100, and 200 ng in PAGE gel (panel A). Protein bands were probed with avidin-AP. Purified 2-12 Tg B cells (2 × 10^6 cells; panel B) or nontransgenic B cells (panel C) were incubated with different amounts of biotin-snRNP D for 1 h. Cells were washed and lysed with lysis buffer. Lysates were subjected to SDS-PAGE, blotted to nitrocellulose, and probed with streptavidin-AP and substrate.

As discussed earlier, autoimmune T cells have been found in the repertoire of normal humans and mouse models. To explore whether snRNP-specific B cells are able to activate autoreactive T cells from the repertoire of non-autoimmune-prone animals, snRNP D protein binding and nonbinding B cells were purified as APCs and cocultured with snRNP-specific CD4 T cells from wild-type B10.A mice. As indicated in Fig. 4A, CD4 T cells from wild-type mice had a profound response to Ag presented by snRNP D binding Tg B cells but not by weaker affinity or non-snRNP D-binding B cells or syngeneic wild-type B cells. The implication of this response is that snRNP-binding Tg B cells process unique snRNP D peptides to which autoreactive T cells have never encountered and thus never tolerated or deleted. In a manner similar to that found in MRL mice (Fig. 2), this experiment demonstrates that autoreactive T cells can also be revealed from the repertoire of a non-autoimmune-prone mouse with BCR-mediated uptake and processing of the self-Ag.

**Discussion**

This study addresses the dynamics of Ag processing and presentation between the known subsets of cells with these functions. Clearly, a number of lymphopoietic cell lineages possess this function, but little is understood of how they may interact in an ongoing immune or autoimmune response. The important role of DCs in this process continues to be studied by many at both the molecular and cellular level as well as in applications to therapeutics by adoptive transfer.

**Other professional APCs activate T cells after the induction of autoimmunity**

We next assessed the potential in vivo role of other APCs, such as DCs that may acquire Ag, in the activation and/or perpetuation of autoreactive T cells. For example, it remained possible that B cells acquired Ag, died, and were taken up by other phagocytic APCs. To evaluate this possibility, we looked for direct evidence of APC capability of B cells and DCs at different time points in vivo. If the conventional model of Ag presentation is correct, DCs would be the first APC to demonstrate T cell activation. Conversely, B cells would not effectively activate T cells. For these experiments, snRNP-specific T cells were used as a readout of Ag presentation by various APC subsets (as demonstrated in Fig. 1B). Two sources of APCs from NP-snRNP immunized mice were used for Ag presentation, either purified B cells or purified DCs. B cells or DCs were obtained from NP-snRNP mice at days 1, 2, 3, 4, and 14 postimmunization.

Either purified B cells or DCs were cocultured with primed snRNP-reactive T cells in the presence or absence of Ag. As indicated in Fig. 5A (without adding Ag), CD4 T cells had a vigorous response to Ag presented by B cells collected from mice from 1 to 4 days after immunization. By day 14, however, B cells had lost their ability to activate T cells. In contrast, purified DCs had a poor capacity to activate CD4 T cells early after in vivo exposure to Ag (purified at day 1). By day 3, DCs acquired the ability to activate CD4 T cells, although B cells were still superior APCs through day 4. By day 14, DCs are the principal APC for stimulating autoreactive T cells. Fig. 5B illustrates that either APC population activate T cells to the same degree when APCs are pulsed with excess self-Ag in vitro. These data indicate that Ag-specific B cells capture self-Ag at the initial phase and only subsequently do DCs acquire APC functions. Most likely, this results from DCs obtaining Ag from B cells, which are known to store it.
Alternatively, Ag-specific B cells can present peptides to T cells at up to 10,000-fold lower Ag concentration compared with non-Ag-specific B cells or other APCs (28, 29). B cells, by virtue of their surface Ig, bind and concentrate Ag from a complex sea of self-Ags, a property that may be relevant in presenting autoantigens that are in low concentration outside of cells in vivo. However, the role of Ag-specific presenting B cells in the activation of naive T cells has remained controversial for a number of years. Indeed, several previous studies have implicated B cells as critical in the induction of T cell tolerance and unresponsiveness (30–35). However, studies from our laboratories and others over the years have illustrated an important role of B cells in the priming and activation of T cells to both foreign and self-Ags (2, 16–22, 36–38). Studies in B cell-deficient autoimmune-prone mice have further demonstrated an important role for the spontaneous activation of T cells by B cells presenting autoantigen in vivo (22). This study differs from others in that we examined the in vivo kinetics over time of either APC subset, B cells or DCs, to activate T cells. Clearly, the presence of both APC subsets in experimental mice would allow either group to present Ag with stoichiometry that mimics the normal induction of immunity.

The use of NP hapten-specific B cells allowed us to carefully control the exposure of normally ubiquitous self-Ags, such as the snRNP autoantigen, to the BCR. Other professional APCs, such as DCs and macrophages, are able to acquire haptenated autoantigen in an otherwise normal manner. Therefore, our experimental system allows one to simultaneously compare the kinetics and Ag presentation mechanisms via receptor-mediated uptake by B cells vs passive uptake of Ag by other professional APCs from the same animal.

Moreover, a lack of secreted Ab in our model system eliminates any interpretation that autoantigen presentation is mediated through soluble immune complexes that may be taken up by FcR of professional APCs. Our use of two independent Ig transgenic mice provides direct evidence that targeting self-Ag to BCRs can activate specific autoantigenic T cells in vitro and in vivo. Importantly, we have also demonstrated that this mechanism can trigger snRNP autoantigenic T cells from the repertoire of a normal, non-autoimmune-prone mouse strain.

One of the new findings from the present work is in addressing how the presence of the self-Ag influences the induction of T cell autoimmunity. NP modification of the snRNP autoantigen was the only means by which B cells could bind and present autoantigen in triggering autoimmune T cell responses. Nonhaptenated snRNP autoantigen failed to elicit autoreactive T cells in recipient mice. The fact that other resident APCs, DCs and macrophages, are present in the repertoire during autoantigen exposure and do not trigger autoimmunity suggest that B cells are a critical first element in the mechanism. In the course of spontaneous human autoimmunity, however, this pathway would require the presence of autoreactive B cells in the normal repertoire, an observation made by a number of other investigators (39, 40). The fact that NP-specific B cells represent only 3% of the transgenic B cell repertoire lends biologic importance to the mechanisms that may be triggering T cells in a normal individual. For example, studies by Nussenzweig and coworkers demonstrate that autoreactive B cell populations inhabit as much as 30% of the normal repertoire; rheumatoid factor-specific B cells constitute from 3 to 5% of the repertoire, frequencies that reflect the autoantigen binding repertoire in our studies (39, 40).

The specificity of the anti-snRNP autoimmune response mediated by NP-specific B cells was further confirmed by demonstrating that autoimmune T cells are activated by an unrelated group of autoantigen-specific B cells (anti-snRNP Ig transgenic B cells) that have never been artificially pulsed with self-Ag either in vivo or in vitro. The latter observation demonstrates that anti-snRNP-specific B cells find and acquire an in vivo source of snRNP autoantigen that can activate autoreactive T cells. Although we could not yet predict the source of in vivo acquired autoantigen, lupus autoantigens have been identified in the serum and other extracellular environments.

More importantly, we demonstrate that other professional APCs activate autoreactive T cells later in the immune response. We find that macrophages and/or DCs acquire self-Ag over longer time periods in vivo (beginning at day 3 or 4 after exposure to self-Ag) and are able to activate primed autoreactive T cells (B. P. Harvey, A. M. Haberman, M. J. Shlomchik, and J. J. Mamula, submitted for publication). At least two explanations may account for these observations. First, professional APCs, DCs and/or macrophages, may naturally need to mature and phagocytose NP-snRNP Ag over time after it is introduced in vivo. This is likely because receptor-mediated uptake via BCR, FcR, CD19, or other receptors, typically occurs more rapidly than passive phagocytic processes (28, 29, 41). This could account for the relatively delayed ability of DCs to activate responding autoreactive T cells. A second mechanism that could explain our observations is the by the direct transfer of Ag.

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**FIGURE 5.** Ag-specific B cells present self-Ag early in the immune response followed by Ag presentation by DCs. CD4 T cells were purified from NP-snRNP D protein immunized anti-NP membrane transgenic BALB/c mice at day 14. NP-transgenic B cells and CD11c⁺ DCs were purified from NP-snRNP D protein immunized anti-NP membrane transgenic BALB/c mice at days 1, 2, 3, 4, and 14. CD4 T cells were cocultured with irradiated NP-transgenic B cells or CD11c⁺ DCs in the absence of any exogenous Ag (A) or in the presence of excess NP-snRNP D protein (5 μg/ml) for 72 h (B). Proliferation was quantified by uptake of [³H]TdR (see Materials and Methods). Data are representative of three independent experiments (three mice/group).
between B cells and DCs. The latter mechanism has been supported by prior studies illustrating several mechanisms by which the transfer of Ag may occur between APC subsets (42–45). Antigenic peptides have been known to be exchanged between APCs via cell-to-cell contact or via gap junctions between cells (42). Finally, Ag-containing exosomes may also be transferred between APCs (44, 45). Preliminary studies from our laboratory support a mechanism whereby cell contact between Ag-presenting B cells and DCs or macrophages transfers antigenic protein and/or peptides (B. Harvey and M. Mamula, submitted for publication). Our data suggest an important role for B cell APC function early in the induction of autoimmunity only if they are present in the repertoire. However, it is likely that autoantigen presentation by specific B cells is normally controlled by their deletion from the repertoire or by other regulatory mechanisms. In this case, other APCs, including DCs and/or macrophages, would then become the first cells to present autoantigens.

Lupus autoimmunity targets a select group of intracellular proteins and peptides among the hundreds of potential intracellular Ags. Cells that may release their many proteins into the extracellular environment would have a similar kinetics of uptake by phagocytosis into DCs or macrophages. The mechanism described in these studies provides one pathway by which autoimmunity may initially focus autoimmunity toward selected intracellular Ags by virtue of uptake through the BCR. As such, this may also contribute to the success of B cell depletion therapies in various autoimmune syndromes (46–48). Moreover, studies from Marshak-Rothstein and colleagues (13, 14) emphasize the importance of cooperative signaling through both BCR and TLR in the induction of lupus autoimmunity. The latter mechanism may also contribute to the mechanisms of how B cells can become both activated by innate mechanisms yet also select specific self-Ags for T cell activation. Our ongoing studies are designed to determine the cell biology of Ag transfer between Ag-specific B cells and other professional APCs. This work also emphasizes the critical role of other APCs in perpetuating the autoimmune response over time.

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Disclosures

The authors have no financial conflict of interest.

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