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Virus-Like Display of a Neo-Self Antigen Reverses B Cell Anergy in a B Cell Receptor Transgenic Mouse Model

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The ability to distinguish between self and foreign Ags is a central feature of immune recognition. For B cells, however, immune tolerance is not absolute, and factors that include Ag valency, the availability of T help, and polyclonal B cell stimuli can influence the induction of autoantibody responses. Here, we evaluated whether multivalent virus-like particle (VLP)-based immunogens could induce autoantibody responses in well-characterized transgenic (Tg) mice that express a soluble form of hen egg lysozyme (HEL) and in which B cell tolerance to HEL is maintained by anergy. Immunization with multivalent VLP-arrayed HEL, but not a trivalent form of HEL, induced high-titer Ab responses against HEL in both soluble HEL Tg mice and double Tg mice that also express a monoclonal HEL-specific BCR. Induction of autoantibodies against HEL was not dependent on coadministration of strong adjuvants, such as CFA. In contrast to previous data showing the T-independent induction of Abs to foreign epitopes on VLPs, the ability of HEL-conjugated VLPs to induce anti-HEL Abs in tolerant mice was dependent on the presence of CD4+ Th cells, and could be enhanced by the presence of pre-existing cognate T cells. In in vitro studies, VLP-conjugated HEL was more potent than trivalent HEL in up-regulating surface activation markers on purified anergic B cells. Moreover, immunization with VLP-HEL reversed B cell anergy in vivo in an adoptive transfer model. Thus, Ag multivalency and T help cooperate to reverse B cell anergy, a major mechanism of B cell tolerance. The Journal of Immunology, 2008, 180: 5816–5825.

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1 Abbreviations used in this paper: HPV; human papillomavirus; VLP; virus-like particle; Tg, transgenic; HEL, soluble hen egg lysozyme; sHEL, soluble form of HEL; HPV, bovine papillomavirus; SA, streptavidin; GMT, geometric mean titer; DblTg, double-Tg mice (Tg[HEL] [mult] sHEL).
coat protein arranged in a T = 3 structure. Presentation of a self-Ag at high density on either platform makes self-Ags as antigenic as a foreign Ag presented in the same context (11–13). These data have raised the possibility that VLP-based vaccines may be effective at inducing Ab responses that could modulate self-Ags involved in chronic diseases, and currently VLP-based vaccines that target angiotensin II (for hypertension), amyloid-β (Alzheimer’s disease), ghrelin (obesity), and TNF-α (psoriasis), among others, are in clinical trials (10, 14).

Unlike simple conjugation to foreign Th epitopes, vaccines to self-Ags based on VLPs elicit high tier anti-self Ab responses at low doses and without the use of adjuvants. By arraying self-Ag on VLPs at different densities, we have shown that the ability to induce autoantibody responses is dependent on high density display of self-Ag (15). Thus, B cell recognition of foreign-like multivalent structural elements, i.e., a dense, repetitive array of Ag, is immunodominant over mechanisms that normally maintain B cell tolerance. However, the mechanism whereby presentation of highly dense, multivalent Ags can activate autoreactive B cells is unclear. It is possible that virus-like display of self-Ags overcomes tolerance primarily at an early stage in the development of autoreactive B cells, before the induction of anergy. Alternatively, interactions with multivalent VLPs may be able to reverse the anergic phenotype of mature autoreactive B cells. IgG responses to Ag are typically initiated only when Ag-specific B cells receive second signals provided by CD4+ T cells. However, some multivalent Ags, including papillomavirus VLPs, are able to activate B cells and induce class switching in the absence of T help (16). In this regard, T-independent responses to pathogens and autoantibody responses to conjugated VLPs are promoted by similar repetitive antigenic structures and are perhaps dependent on similar stringent requirements for Ag-induced cross-linking of BCRs (15, 17, 18). However, it is unclear whether the ability of repetitive Ags to break self tolerance is also T independent.

To better understand the factors responsible for breaking B cell tolerance by VLP displayed self-Ags, and specifically to examine the correlates of overcoming anergy, we examined the parameters underlying autoantibody induction by conjugated VLPs using two well-characterized strains of transgenic (Tg) mice developed by Goodnow et al. These mice include transgenic Ig (TgIg) mice that express a rearranged BCR that binds hen egg lysozyme (HEL). Greater than 95% of the B cells that are produced by TgIg mice are HEL specific. In addition, we used Tg HEL mice that express a soluble form of HEL as a neo-self Ag (sHEL mice). In previous studies, these mice did not respond to HEL immunization, even when HEL was linked to a source of foreign Th epitopes, such as SRBCs (19). Double-Tg mice (TgIg × sHEL; DbTg) express both transgenes. The B cells from DbTg mice are monospecific for HEL but have a classic anergic phenotype and did not produce HEL-specific Abs in response to immunization with SRBC-HEL (19).

In this study, we examine the relative roles of Ag valency and T help in the induction of HEL-specific Ab responses in tolerant (sHEL and DbTg) and nontolerant (C57BL/6 and TgIg) mice. Our data show that virus-like display reverses B cell anergy in the HEL model and indicate that both Ag valency and the availability of T help play critical roles in this process.

Materials and Methods

Mice

Inbred C57BL/6 mice were obtained from Harlan Breeders. Mice Tg for sHEL and Tg mice that express I and L Ig chains specific for HEL (TgIg mice), originally generated by Goodnow et al. (20), were obtained from the Jackson Laboratory. DbTg mice expressing the transgenic Ig and sHEL were obtained by crossing female sHEL mice with male TgIg mice. Because B cells expressing the transgenic Ig were IgMδGδ* whereas endogenous B cells were IgMδGμ*, offspring were screened for the presence of the TgIg transgene by flow cytometry using FITC-labeled mAbs against IgD* or IgM* (both BD Pharamingen). TgIg mice were IgD* IgMδGδ(*/+) and DbTg mice were IgD* IgMδGδ(*/++). Mice were typically 4–8 wk old when used in experiments. All animal care was in accordance with the National Institutes of Health and University of New Mexico guidelines.

Generation of HEL-conjugated VLPs

HPV-16 VLPs and GFP-labeled bovine papillomavirus type 1 (BPV-1) VLPs were made and purified as described (21, 22). Briefly, HPV-16 VLPs were generated by transfecting 293T cells with an expression vector containing the HPV-16 major capsid protein L1. GFP-labeled VLPs were made by cotransfecting 293T cells with plasmids encoding BPV-1 L1 and a BPV-1 L2-GFP fusion protein (23). VLPs were purified 48 h posttransfection by centrifugation through an Optiprep (Sigma-Aldrich) gradient. QB bacteriophage was a gift from Dr. David Peabody.

Purified VLPs were biotinylated by incubation with N-hydroxysuccinimidyl-long chain-biotin (Pierce Endogen) at a 1:2 (w/w) ratio for 60 min at room temperature. Excess unconjugated biotin was removed, and biotinylated VLPs were concentrated by centrifugation and buffer exchange using the Amicon-Ultra-4 centrifugal filter device (Millipore) with a 100-kDa limit. HEL-conjugated particles were generated by linking biotinylated HEL (Biomeds) to biotinylated VLPs using streptavidin-SA (SA; Zymed). Biotinylated HEL was reacted with SA at a 1:1 mole:mole ratio overnight at 4°C. This material, designated SA-HEL, was added to biotinylated VLPs at a 1:1 (µg of SA:µg of HPV-16 L1) ratio at 4°C overnight to generate HEL-conjugated VLPs (VLP-HEL).

Vaccinations

Mice were inoculated with HEL-conjugated VLPs, SA-HEL, or SA, as described in the text. If CFA was used, Ag was diluted 1/1 in CFA (first injection) or IFA (subsequent inoculations). In some cases, IFA was used for all three inoculations. Typically, mice were inoculated i.m. three times at 2-wk intervals. Serum samples were collected at each inoculation and 1–2 wk after the final boost.

Quantitating Ab responses

Ab titers were determined by end-point dilution ELISA. Immulon II ELISA plates (Dynex Technologies) were coated with 200–500 ng of Ag (HEL or SA) diluted in PBS in a total volume of 50 µl overnight at 4°C. The ELISA was performed as described previously (12). Briefly, after blocking wells with PBS, 0.5% milk (blocking buffer) mouse serum was serially diluted in blocking buffer and applied to wells for 2.5 h at room temperature. Reactivity to target Ag was determined by using HRP-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a 1:2000 dilution in blocking buffer; HRP-labeled goat anti-mouse IgM (Zymed) was used for all three inoculations. Typically, mice were inoculated i.m. three times at 2-wk intervals. Serum samples were collected at each inoculation and 1–2 wk after the final boost.

CD4 cell depletion

CD4+ T cells were depleted by treating mice with an anti-CD4 mAb GK1.5. The GK1.5 hybridoma was obtained from the American Type Culture Collection. Ab was prepared from hybridoma supernatants. Hybridoma supernatant was diluted 1/1 in PBS (pH 7.4); then Ig was concentrated by ammonium sulfate precipitation. The solution was brought up to 75% ammonium sulfate by addition of solid. After 2 h at 4°C, the sample was spun at 3000 rpm for 15 min. After centrifugation, the pellet was resuspended in PBS and then mixed with an equal volume of saturated ammonium sulfate. After 1 h at 4°C, the sample was centrifuged, and the resultant pellet was resuspended in a small volume (5 ml) of PBS. After extensive dialysis against PBS, GK1.5 was purified using protein G-coated beads (Pierce Endogen). Groups of mice were injected with 0, 10, 25, or 100 µg of GK1.5 3 days before each immunization. The efficiency of T cell depletion was assessed by flow cytometric analysis of peripheral blood using a FITC-labeled anti-CD4 Ab (RM4-5; BD Pharamingen), which binds to a region of CD4 that is distinct from the GK1.5-binding site.
B cell culture

B cells were isolated from mouse splenocytes by using one or two rounds of negative selection using magnetic microbeads coupled with Abs against mouse CD11b and CD43 (Miltenyi Biotec). B cell purity was measured by flow cytometry, using a FITC-labeled anti-B220 Ab (RA3-6B2; BD Pharmingen). After negative selection, cells were 90–95% B220+. Cells were cultured in B cell medium (RPMI with 10% heat-inactivated FCS, 2 mM l-glutamine, 55 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin) at a concentration of 10^6 cells/ml. After stimulation with HEL, B cells were monitored by flow cytometry using FITC-labeled mAbs against CD69, CD40, and CD86 (all BD Pharmingen).

Adaptive transfers

Donor B cells were isolated from the spleens of TgIg or DblTg mice by negative selection as described in B cell culture. Recipient nontransgenic (C57BL/6) or Tg (sHEL) mice were injected i.v. via the retro-orbital cavity with 8 X 10⁶ B cells in a total volume of 0.2 ml. One day after the adoptive transfer, mice were immunized i.m. with 10 μg of HEL-conjugated VLPs with iFA. Mice were boosted with another 10-μg dose 2 wk later. Serum samples were collected 1 wk after each immunization, and anti-HEL IgM Ab responses were measured by ELISA.

Results

HEL-conjugated VLPs bind to the Tg HEL-specific BCR

B cells that are specific for self-Ags normally do not respond to antigenic stimulation when in a tolerizing environment. However, we have shown that the arrangement of self-Ags in the context of a dense, repetitive array, such as on the surface of a virion or VLP is highly immunogenic and can induce Ab responses against self. To determine whether VLP conjugation could induce anti-HEL autoantibodies in the well-characterized HEL mouse model of B cell tolerance, we first generated a multivalent Ag in which HEL was conjugated at high density to the surface of VLPs. These HEL-conjugated VLPs were generated using the SA-bridge approach, which we have previously shown results in high-density coating of VLPs with target Ag (24). Biotinylated HEL was reacted with SA at a molar ratio that leaves, on average, at least one of the four biotin-binding sites on the SA tetramer unoccupied. The SA-HEL conjugate was subsequently conjugated to biotinylated papillomavirus VLPs. Analytical centrifugation on an Optiprep gradient showed that HEL cosedimented with the papillomavirus major capsid protein, L1, in the higher-molecular-mass fractions of the gradient, confirming that the HEL was bound to the biotinylated VLPs (data not shown). Using the same SA-bridge technique, we also conjugated HEL to another Ag platform, Qb bacteriophage (data not shown). One advantage of this conjugation technique is that we could also use an intermediate of the conjugation reaction, SA-HEL. SA-HEL-SAHEL is, on average, a trivalent form of HEL that, by virtue of linkage to SA, contains Th epitopes that are sufficient to provoke high-titer anti-SA IgG responses upon immunization of C57BL/6 mice (12).

To ensure that VLP-conjugated HEL bound to the transgenic BCR expressed on the surface of B cells from TgIg mice, we conjugated HEL to VLPs that contained GFP and then examined the binding of the labeled HEL-conjugated particles to TgIg B cells. GFP-labeled HEL-VLPs were incubated with splenocytes from TgIg mice, and binding to specific cell types was measured by flow cytometry. GFP-labeled HEL-VLPs bound specifically to B220⁺ TgIg splenocytes (Fig. 1). Preincubation of cells with HEL substantially reduced HEL-VLP binding, indicating that VLP-HEL binding to the B cells was mediated by HEL. The inability to completely block HEL-VLP binding may reflect the higher avidity that multivalent HEL on VLPs is likely to have for the TgIg BCR relative to monomeric HEL. HEL-VLP did not bind to either CD3⁺ or CD11b⁺ cells isolated from the spleens of TgIg mice or to B220⁺ cells from control C57BL/6 mice (data not shown).

These data indicate that conjugation to VLPs does not impair the ability of HEL to react with the HEL-reactive TgIg BCR.

HEL-conjugated VLPs induce anti-HEL Ab responses in both tolerant and nontolerant mice

To examine the role of valency in influencing the ability to induce Ab responses against HEL in tolerant and nontolerant mice, we immunized mice with trivalent HEL (linked to SA; SA-HEL) or multivalent HEL (SA-HEL conjugated to VLPs; VLP-HEL). Groups of mice were immunized with three doses of biotinylated HEL (11.25 μg) linked to SA (15 μg), or the same dose of SA-HEL conjugated to 5 μg of HPV16 VLPs (VLP-HEL) or 5 μg of Qb bacteriophage VLPs (Qb-HEL). Ab responses were measured by end-point dilution ELISA.

First, we examined the relative ability of multivalent and trivalent HEL to induce Ab responses in tolerant and nontolerant mice with a normal B cell repertoire. In nontolerant C57BL/6 mice, both SA-HEL and VLP-HEL elicited high titer anti-HEL Ab responses. The anti-HEL IgG geometric mean titer (GMT) was higher (~10-fold) in mice immunized with VLP-HEL compared with SA-HEL (Fig. 2A). The effects of VLP conjugation on anti-HEL Ab responses in tolerant sHEL Tg mice were more substantial. Whereas IgG Ab responses in sHEL mice immunized with SA-HEL (plus CFA) were weak (GMT = 40), VLP-HEL immunized mice had an anti-HEL IgG GMT that was >60 times higher (GMT = 2560). Anti-HEL IgG responses upon immunization with VLP-HEL were similar when either CFA or IFA was used. IgG responses against SA, a foreign Ag, were only slightly (~4-fold) higher in HEL-VLP-immunized mice (SA-HEL-immunized mice, GMT = 2560; VLP-HEL-immunized mice, GMT = 10,240). The ability to mount a strong IgG response against SA indicates that linked T help was not limiting in the mice immunized with SA-HEL. Thus, presentation of HEL on VLPs abolishes any immune distinction between self and foreign Ag, whereas simple conjugation to a source of foreign Th epitopes does little to abolish differences in immune responsiveness.

To examine whether the ability to induce anti-HEL responses was applicable to another virus platform, we also tested whether HEL-conjugated Qₘ bacteriophage could induce anti-HEL responses in sHEL Tg mice (Fig. 2A). HEL-conjugated Qₘ (plus

FIGURE 1. HEL-conjugated VLPs bind to B cells from TgIg mice. GFP-containing HEL-conjugated bovine papillomavirus VLPs were generated as described in Materials and Methods. Freshly isolated splenocytes from TgIg mice were adjusted to 1 X 10⁶ cells/ml in PBS, 0.1% BSA, 0.1% azide and reacted with (bold histogram) or without (normal histogram) GFP-HEL-VLPs at a concentration of 10 μg/ml plus a PE-labeled anti-B220 mAb (BD Pharmingen) for 30 min on ice. After two rounds of washings, cells were subjected to flow cytometry. In some cases, cells were preincubated with HEL at a concentration of 10 μg/ml (dashed histogram). Histograms show mean fluorescence intensities as a measure for GFP-HEL-VLP binding and are gated for B220⁺ cells.
IFA) generated extremely-high-titer anti-HEL IgG responses in sHEL Tg mice. The anti-HEL GMT was >1000-fold higher than in mice immunized with SA-HEL. HEL-conjugated Qβ phage also induced high titer anti-HEL IgG in the absence of exogenous adjuvant. It is possible that encapsidated viral RNA (with TLR agonist properties) could account for the immunogenicity, relative to the papillomavirus VLP carrier, in the absence of exogenous adjuvant or in the presence of a simple depot adjuvant such as IFA. Thus, multivalent display of HEL on both Qβ phage and HPV VLPs elicited high-titer IgG responses against HEL in tolerant sHEL Tg mice.

Ab responses against HEL were next measured in an anti-HEL Ig-transgenic mouse model (Fig. 2B). TgIg mice express transgenic H and L Ig chains specific for HEL, and >95% of their B cells are specific for HEL. Because of the nature of the transgene, B cells from TgIg and DblTg mice fail to undergo class switch recombination and therefore only express IgD and IgM on their surface and only secrete IgM. Therefore, Ab responses against HEL were assessed by measuring anti-HEL IgM levels by ELISA. In our hands, spontaneous secretion of anti-HEL IgM in unimmunized TgIg mice resulted in background serum titers ranging from 160 to 640 (Fig. 3B). When TgIg mice are crossed with sHEL Tg mice, the resulting DblTg mice express B cells with a classic anergic phenotype. No anti-HEL IgM above background levels were detected in the sera from unimmunized DblTg mice (Fig. 3D).

Not surprisingly, immunization with both HEL-conjugated VLPs and SA-HEL resulted in high-titer anti-IgM responses in nontolerant TgIg mice (Fig. 2B). However, only multivalent HEL (VLP-HEL and Qβ-HEL) induced high-titer IgM responses against HEL in tolerant DblTg mice (Fig. 2B). In the presence of adjuvant (either CFA/IFA or IFA alone), both VLP-HEL and Qβ-HEL induced IgM levels that were 102- to 103-fold higher than in mice immunized with SA-HEL. In the absence of adjuvant, SA-HEL failed to induce detectable anti-HEL IgM, but both Qβ-HEL and VLP-HEL induced anti-HEL IgM responses (GMTs of 640 and ~100, respectively). A higher dose of Ag (20 μg vs 5 μg of
VLP-HEL (Fig. 4). For example, a single immunization with VLP-HEL or QA produced extremely low IgM titers (detectable at a 1/40 dilution in only two of five mice). Subsequent boosts with SA-HEL resulted in increased (∼10-fold) IgM levels, suggesting that adjuvant may function by increasing the local concentration of Ag and this effect may be mimicked by an increase in Ag dose. Thus, these data show that multivalent presentation of HEL on papillomavirus VLPs or QA phage overcome the effects of anergy in the HEL mouse model.

Effects of boosting in Ab induction

The kinetics of Ab induction was examined by measuring anti-HEL titers in tolerant and nontolerant mice after each immunization. Sera were taken before immunization and 1 wk after each immunization, and then HEL-reactive Ab titers were measured by ELISA. As before, we examined IgM responses in TgIg and DblTg mice with a monospecific B cell population and IgG responses in wild-type and sHEL Tg mice that have a normal B cell repertoire (Fig. 3). In nontolerant C57BL/6 and TgIg mice, these data reiterate the potent immunogenicity of multivalent HEL (Fig. 3, A and B). For example, a single immunization with VLP-HEL or QA-HEL resulted in anti-HEL IgM titers of >10^4 (Fig. 3B). A subsequent boost resulted in a 10-fold increase in the IgM titer. Immunization with SA-HEL resulted in a less substantive primary IgM response (GMT = 640), which is not significantly above the normal background level of HEL-specific IgM. Subsequent boosts ultimately resulted in similar IgM levels as measured in mice immunized with multivalent VLP-HEL.

In tolerant DblTg mice, a single immunization with VLP-HEL or QA-HEL resulted in low, but measurable (at least 40) anti-HEL IgM responses in all 10 immunized mice that we tested (Fig. 3C). These Ab titers were considerably lower (>100-fold) than that observed in the TgIg mice. Measurable anti-HEL Ab in DblTg mice may have been decreased by the presence of sHEL in the serum, which is expected to bind to a percentage of the HEL-specific IgM. It is also possible that it may simply take more than 7 days for anergic B cells to become fully activated and secrete enough Ab to make a substantial difference in serum titers. A single booster injection resulted in high IgM titers in these mice (GMT ∼10^6 after two injections) with titers of 640 or higher in 9 of 10 mice. In contrast, a single immunization with SA-HEL resulted in extremely low IgM titers (detectable at a 1/40 dilution in only two of five mice). Subsequent boosts with SA-HEL resulted in only small increases in the IgM titer (only one of five mice had a titer of >40 after two injections, and only two of five had titers of >40 after three injections). The kinetics of Ab induction in sHEL mice immunized with HEL-VLPs followed a pattern similar to that observed in DblTg mice (Fig. 3D), although because IgG secretion is dependent on class switching, anti-HEL IgG were first observed after two immunizations. sHEL mice were largely unresponsive to immunization with SA-HEL (GMT = 15 after two injections, whereas after two immunizations with HEL-VLP titers were >10 times higher (GMT = 225). Thus, the multivalent form of HEL is highly immunogenic in both tolerant and nontolerant settings.

Anti-HEL IgG responses are dependent on T help

We next examined the role of T cells in the induction of HEL-specific IgG in sHEL mice that were completely or partially depleted of CD4+ T cells. Before immunization, mice were injected with GK1.5, a rat anti-mouse CD4 mAb that depletes CD4+ T cells in vivo. Groups of sHEL mice were injected with different doses of GK1.5 (0, 10, 25, or 100 μg) to deplete CD4+ T cells to different extents. These doses have previously been shown to lead to reductions in CD4+ T cells ranging from 50% (10 μg) to >90% (100 μg) (25). Three days after each injection, CD4+ T cell levels were determined by flow cytometry. Weekly injections of GK1.5 maintained the lowered CD4+ T cells levels (data not shown).

Each group of CD4-depleted mice was immunized with VLP-HEL (plus IFA) two times with a 2-wk interval, and Ab responses against self (HEL) and foreign (SA) components of the vaccine were measured by ELISA. Because the extent of CD4 depletion was variable in each group of GK1.5-treated mice, Ab titers were examined relative to the extent of CD4+ T cell depletion; full depletion (>80%), partial depletion (50 ± 10%), or no depletion (not treated with GK1.5). One week after immunization with a single dose of VLP-HEL, all of the mice had detectable IgM Abs against HEL, although IgM levels were lower in mice subjected to CD4 depletion (Fig. 4A). After two immunizations, we were unable to detect IgG responses against either SA or HEL in the three mice in which CD4+ T cells were >80% depleted (Fig. 4, B and C). These data indicate that in
mice tolerant to one component of the immunogen, induction of IgG using conjugated VLPs is T dependent for both the tolerogen and linked foreign epitopes. Relative to untreated mice, partial depletion of CD4⁺ cells had little effect on IgG responses to either the self or foreign components of the immunogen. Therefore, there may be a threshold of CD4⁺ T cells required to mount an IgG response against HEL-VLPs.

**Enhancement of Ab responses by pre-existing T help**

Taken together, our results indicate that both Ag valency and the availability of T help play important roles in the induction of Ab responses against self Ags. Next, we asked whether pre-existing activated Th cells could enhance Ab responses to trivalent or multivalent HEL in tolerant or nontolerant mice. Although HEL cannot elicit a Th response in the C57BL/6 background (26), SA, which is a component of both SA-HEL and VLP-HEL, can elicit robust Th responses (12). Therefore, TgIg or DblTg mice were primed by i.m. injection of 100 μg of SA in CFA. After 1 wk, mice were immunized with VLP-HEL or SA-HEL in IFA, and Ab responses were measured 1 wk later. Responses were compared with mice that were not primed with SA (○, ■).

In nontolerant TgIg mice, priming with SA had only a slight boosting effect (~2-fold) on anti-HEL Ab levels measured in mice immunized with VLP-HEL (Fig. 5A). Priming resulted in a much more substantial increase in anti-HEL IgM levels (25-fold) in mice immunized with SA-HEL; IgM titers were equivalent to those measured in mice immunized with VLP-HEL. Therefore, in nontolerant mice, the presence of pre-existing Th cells specific for SA enhanced Ab production to the same extent as multivalency.

We also examined the effects of SA priming on Ab responses in tolerant DblTg mice (Fig. 5B). SA priming resulted in a modest boost in the anti-HEL IgM titer upon immunization with SA-HEL (~3.5-fold) to a titer that was equivalent to that observed in unprimed mice immunized with VLP-HEL. Priming with SA also had a substantial effect on titers in mice immunized with VLP-HEL (~8-fold). Unlike TgIg mice, the availability of pre-existing Th help did not bring the Ab titers upon SA-HEL immunization to levels similar to those of mice immunized with VLP-HEL. (VLP-HEL mice were 5.5-fold higher, p < 0.05). These data indicate that in a tolerizing environment both the availability of T help and multivalency play important roles in the induction of autoantibody responses.

**In vitro stimulation of transgenic B cells with HEL at different valencies**

One important feature of the HEL Tg mouse system is the ability to purify homogeneous populations of HEL-specific B cells from the spleens of nontolerant (TgIg) or tolerant (DblTg) mice. We used these cells to examine, in vitro, how Ag valency and dose influence B cell activation. B cells were purified by negative selection from splenocytes isolated from nontolerant TgIg and tolerant DblTg mice and were then stimulated with different amounts (ranging from 1 to 2000 ng/ml) of VLP- or SA-conjugated HEL. After 24 h, the relative expression of surface markers associated with B cell activation and subsequent interactions with Th cells were measured by flow cytometry. We measured expression of three B cell surface markers: CD69, an early B cell activation marker; CD86, a costimulatory molecule that interacts with CD28 on Th cells; and CD40, which interacts with CD40L on Th cells and is a key player in promoting B cell proliferation and in triggering the germinal center reaction. Results are shown in Fig. 6 as geometric mean fluorescence intensity relative to unstimulated (no HEL) controls. As a control, B cells isolated from non-Tg C57BL/6 mice were cultured with VLP-HEL. Control non-Tg B cells did not up-regulate surface expression of any of the markers we examined (Fig. 6), indicating that conjugated VLPs do not stimulate B cells in a BCR-independent manner.

Nontolerant TgIg B cells were stimulated with VLP- and SA-conjugated HEL at concentrations that ranged from 1 to 1000 ng/ml. At equivalent HEL concentrations, TgIg B cells responded more vigorously to stimulation with HEL-VLPs than SA-HEL (Fig. 6). CD69 expression peaked at ~10 ng/ml HEL-VLPs. An equivalent level of CD69 expression was only observed at 200 ng/ml SA-HEL (Fig. 6A). CD86 expression was also induced more strongly and at lower doses by VLP-HEL (Fig. 6B). CD40 expression was also up-regulated to a greater extent by multivalent HEL, although the magnitude of increased expression was less substantial, perhaps due to the fact the CD40 is constitutively expressed on B cells (Fig. 6C). Thus, TgIg B cells responded equivalently to a HEL concentration on VLPs that was roughly 10- to 100-fold lower than when stimulated with trivalent SA-HEL.

DblTg B cells express ~10- to 30-fold less surface IgM than TgIg B cells (27) and respond poorly to stimulation with soluble HEL (19, 28). Given that our data indicated that VLP-HEL could induce anti-HEL Ab responses in DblTg mice, we tested whether VLP-HEL could stimulate DblTg B cells, and compared these responses to DblTg B cells stimulated with trivalent SA-HEL. As shown in Fig. 7, at the highest concentration of HEL tested (2000
ng/ml), stimulation with VLP-HEL resulted in higher expression of CD69, CD86, and CD40 than stimulation with SA-HEL. The increase of expression of these markers were observed in the bulk population of B cells, and thus do not represent activation of only a possible subset of nonanergic cells. In general, DblTg B cells responded more strongly to varying concentration of multivalent HEL on VLPs than to trivalent SA-HEL (Fig. 6). However, DblTg B cells were much less sensitive (≈500-fold) to stimulation with VLP-HEL than what we observed when using TgIg B cells. DblTg B cells expressed higher levels of CD69 and CD86 upon stimulation with VLP-HEL than with similar concentrations of SA-HEL, although CD69 up-regulation was not pronounced except at the highest dose tested. Notably, stimulation of DblTg B cells with SA-HEL did not result in any measurable increase in CD40 expression, whereas stimulation with VLP-HEL resulted in modest but reproducible increases in CD40 expression. Thus, these data indicate that both tolerant and nontolerant B cells respond to multivalent HEL and that increased Ag valency leads to quantitative boosts in the expression of phenotypic activation markers.

The anergic state of DblTg B cells can be reversed in vivo by VLP-HEL immunization

Our data are consistent with the hypothesis that highly multivalent Ags can reverse the effects of anergy. However, it remains formally possible that multivalent Ags can induce anti-HEL Abs by stimulating newly emerged B cells from the bone marrow that have not yet been anergized. To test the ability of VLP-HEL to directly stimulate anergic B cells in vivo, we performed adoptive transfer experiments in which mature transgenic B cells were isolated from the spleens of nontolerant (TgIg) or tolerant (DblTg) mice and then transferred into nontolerant (C57BL/6) or tolerant (sHEL) recipients. To ensure the presence of T help, recipient mice were immunized with 100 µg of SA plus CFA 1 wk before the adoptive transfer. Recipient mice were immunized with 10 µg of VLP-HEL plus IFA 1 day after the transfer and then boosted with the same dose of Ag 2 wk later. Anti-HEL IgM responses were measured by ELISA. Because the donor and recipient mice express different IgM allotypes, anti-HEL IgM produced by the donor B
cells could be distinguished from the endogenous response by using an IgM* specific secondary Ab. No anti-HEL IgM* were detected in immunized sHEL mice that did not receive B cells from a transgenic donor (Fig. 8).

As a positive control, we examined the responsiveness of nonanergic transgenic B cells transferred into nonanergic (C57BL/6) recipients. These B cells responded robustly to immunization with VLP-HEL, resulting in high anti-HEL IgM* titers (GMT 3600) after a single immunization (Fig. 8). We also detected anti-HEL Abs produced by nonanergic transgenic (TgIg) B cells transferred into tolerant (sHEL) recipients (GMT 140) and by anergic (DbITg) B cells transferred into tolerant mice (GMT 170). Although these responses were moderate after a single immunization, they increased >10-fold upon boosting. The responsiveness of transferred TgIg and DbITg B cells in sHEL mice were similar, suggesting the possibility that donor TgIg B cells may have been rapidly anergized upon transfer to the tolerant recipients. Therefore, these results demonstrate that VLP-HEL can reverse the effects of anergy in vivo.

Discussion

Our laboratory and others have demonstrated that highly dense, multivalent Ags are extremely immunogenic and have the ability to induce high titer Ab responses against both foreign and self-Ags. Here, we extended these observations by examining the correlates of autoantibody induction using the well-characterized HEL mouse model of B cell tolerance. These studies were facilitated by the availability of tolerant and nontolerant transgenic mice with monospecific and normal B cell repertoires and the ability to modulate Ag valency and the availability of T cell help. Taken together, these studies demonstrate that highly dense multivalent Ags based on VLPs can readily reverse B cell anergy in a T cell-dependent manner.

In nontolerant C57BL/6 and TgIg mice, multivalent HEL, linked to papillomavirus VLPs or Qb bacteriophage VLPs, induced higher titer anti-HEL Ab responses than mice immunized with trivalent HEL, linked to SA, demonstrating the relationship between Ag valency and Ab production first observed by Dintzis et al. (9). TgIg mice responded vigorously to a single injection with VLP-HEL, whereas multiple immunizations with SA-HEL were necessary to elicit anti-HEL IgM titers at an equivalent level. At a molecular level, these differences in ability to induce Abs corresponded with a hyperresponsiveness of B cells to multivalent HEL relative to B cells stimulated in vitro with equivalent amounts of HEL conjugated to SA. TgIg B cells stimulated with VLP-HEL had increased expression of a B cell activation marker (CD69) and of molecules involved in activating (CD86) and receiving help from (CD40) CD4* T cells. The results of previous studies using anti-IgM Abs of varying valencies (29, 30) are consistent with our results using multivalent Ags. Extensive cross-linking of the BCR by multivalent Ags induces the association of the BCR with detergent-insoluble lipid rafts (31) that are associated with long-term stable activation of tyrosine phosphorylation (32). The ability of multivalent Ags to establish more stable signaling domains is likely to be responsible for the quantitative boost in up-regulation of markers associated with B cell activation upon stimulation with multivalent HEL on VLPs.

The effects of VLP conjugation on Ab induction in tolerant mice were more substantial. In DbITg mice, immunization with VLP-HEL readily reversed the effects of anergy, and induced IgM levels
that were 100- to 1000-fold higher than mice immunized with trivalent SA-HEL. These results show, for the first time, the induction of anti-HEL Ab responses in this model of anergy without the use of polyclonal B cell stimuli, such as CpG oligonucleotides or LPS, or adoptively transferred transgenic HEL-specific Th cells, as previously studies have used (28, 33, 34). CFA was not required to induce anti-HEL responses in our studies; anti-HEL Ab responses could be induced by HEL-conjugated VLPs without exogenous adjuvant.

DbiTg B cells express low levels of surface associated IgM, which allows them to survive, albeit suboptimally, in the presence of soluble HEL (35). Some have expressed skepticism that the decreased levels of surface IgM on anergic B cells accounts for their phenotype (36). However, our data showing that multivalent Ags can rescue anergic B cells indicate that the valency of surface interactions between receptor and Ag play an important role in shaping B cell responsiveness in anergic cells. DbiTg B cells responded more strongly in vitro stimulation with VLP-HEL than to SA-HEL, although, not surprisingly, the up-regulation of the surface markers that we tested was less substantial than we observed using TgIp B cells. Moreover, our adoptive transfer studies showed that immunization with VLP-HEL could reverse the effects of anergic phenotype of DbiTg splenic B cells in vivo. These data formally demonstrate that VLP conjugates can directly reverse anergy and elicit autoantibody production rather than simply activating B cells that have not yet been anergized by soluble HEL.

Stimulation with SA-HEL did not up-regulate CD40 expression on DbiTg B cells, whereas stimulation with VLP-HEL resulted a modest, but reproducible, up-regulation of CD40. The interaction between CD40 on B cells and CD40L on T cells is critical for B cell proliferation and differentiation; therefore, an increase in CD40 expression could make B cells more receptive to T cell help. As a corollary, even small increases in CD40L expression on T cells can have strong effects on Ab production (37). The ability to activate B cells that express low levels of surface Ig is relevant not only in anergic B cells but also potentially relevant to self-reactive cells that have undergone receptor editing. It has recently been demonstrated that B cells that undergo receptor editing continue to express low levels of the autoreactive BCR, through a process that is referred to as allelic inclusion (38). It would be interesting to test whether multivalent Ags could activate subdominant self-reactive BCRs. VLP display has been used to induce autoantibodies against diverse targets to which tolerance may be maintained by anergy and/or receptor editing. These include soluble molecules (such as TNF-α, angiotensin II, and IL-17) as well as membrane-associated self-Ags, such as CCR5 (11, 12, 39–43).

Although the DbiTg mouse model has played an important role in studying the regulation of B cell responsiveness, the physiological relevance of this model is diminished by several features. First, due to the nature of the TgIp transgene, DbiTg B cells do not class switch, and therefore it is difficult to assess late events in B cell differentiation using this model. Second, the B cell population in these mice is monospecific. It may be easier to overcome anergy in this system due to the high frequency of HEL-specific B cells and the lack of competing nonanergic B cells which can lead to follicular exclusion of anergic cells and their reduced survival (6). Third, the transgenic TgIp BCR has extremely high affinity for HEL. The affinity of the BCR for its cognate epitope clearly can influence B cell responsiveness (44) and could conceivably influence how anergy, and activation from an anergic state, are manifested. For all of these reasons, it was also important to demonstrate that VLP display is also effective at overcoming anergy in shHEL mice. Indeed, immunization with VLP-HEL and Q8-HEL induced high titer anti-HEL IgG responses that were >100-fold higher than in mice immunized with SA-HEL. Furthermore, in our adoptive transfer experiment, we showed that VLP-HEL could activate transgenic B cells in a background of normal B cells (in shHEL mice). Thus, we feel that this provides further evidence that VLP-conjugation is an effective method for inducing Ab responses against self. In the ML5 shHEL mice that we used, shHEL is expressed at serum levels of ~20 ng/ml. It would be interesting to determine whether even higher expression of HEL adversely affects the ability to induce autoantibodies using conjugated VLPs.

Many VLP types can induce IgG Abs in a T cell-independent manner. HPV16 VLPs, for example, can directly activate B cells in a TLR4-dependent manner to induce CD4<sup>+</sup> T cell-independent class switching (16). In contrast, our data show that T help is strictly required for the induction of both anti-self and anti-foreign Abs by VLP-HEL. Previously, we showed that conjugated papillomavirus VLPs, unlike wild-type VLPs, fail to directly induce the acute activation of dendritic cells (15). Thus, it seems likely that the failure to induce T-independent responses is a consequence of HEL-conjugation, which prevents the VLP surface from interacting with TLR4. VLPs have been modified so that they encapsidate CpG oligonucleotides, which can interact with TLR9 on B cells (45). It is possible that CpG-containing HEL-VLPs may act as a T-independent Ag. Such particles would also be useful in measuring the quantitative contribution of BCR-independent B cell stimuli in overcoming anergy by conjugated VLPs.

Not only is CD4<sup>+</sup> T help required, but preactivated T cells to one component of the HEL-conjugated VLPs enhance Ab responsiveness upon VLP-HEL immunization. Therefore, these data indicate that both Ag valency and the availability of T cell help play quantitative roles in the induction of Ab responses. For foreign Ags, these factors are important in determining the magnitude of the Ab response. For self-Ags, Ag multivalency and the availability of T help are critical factors that modulate the ability to induce anti-self responses in the face of B cell anergy.

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**References**


