A Novel Mechanism for the Immunomodulatory Functions of Class II MHC–Derived Peptides

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Abstract. There is now extensive evidence that synthetic peptides corresponding to linear sequences of MHC molecules are effective immunoregulators, targeting the immune response at many different sites. It has been previously shown that peptides derived from a highly conserved region of MHC class II inhibit proliferation to autoantigen and to both the direct and indirect pathways of allorecognition. This study demonstrates that inhibition of lymphocyte proliferation by nonpolymorphic MHC class II peptides, specifically HLA-DQA1, is sequence-specific and that the inhibitory effect is mediated through the induction of apoptosis in antigen-presenting cells via a caspase-independent mechanism. In addition, T lymphocytes stimulated in the presence of HLA-DQA1 are rendered hyporesponsive to subsequent stimuli. Immunomodulation by HLA-DQA1 is effective in vivo because it prevents both the priming and the effector function of primed allogeneic T cells in a murine DTH model. These observations have important implications for the development of a novel therapy for immune-mediated diseases.

The adaptive immune response is dependent on the TCR recognition of a peptide ligand complexed with a MHC molecule on the surface of an antigen-presenting cell. This peptide, and the resultant three-dimensional structure formed by it and the MHC molecule, interacts with the TCR triggering a series of signaling events resulting in T cell activation (1). Critical residues within the peptide form the contact points with the TCR, and variation at one of these sites alone is sufficient to disrupt the interaction or change the T cell response qualitatively, resulting in differential cytokine production, antagonism, or anergy (2). There are now several reports of altered peptide ligands that induce partial activation in vitro, resulting in altered responses to autoantigens and allergens (3,4). In addition, the immunoregulatory effects of altered TCR ligands has been demonstrated in vivo, as exemplified by the prevention of experimental autoimmune encephalomyelitis (5). Such studies require that the specific antigen that elicits the immune response be identified. The pivotal role of peptides in allorecognition has highlighted them as a potential strategy for altering the alloimmune response (6,7). Difficulty in identifying the critical allopeptides recognized by alloreactive T cell clones for each given donor-recipient combination make the use of altered peptide ligands potentially less clinically applicable in transplantation (8).

APC are continually presenting antigens on the cell surface that represent molecules within their environment, including self molecules. Indeed, it has been demonstrated that a large percentage of peptides bound to MHC on resting APC are derived from MHC molecules themselves (9). The presence of these MHC-bound peptides derived from conserved regions of the MHC raises questions as to their role in the immune process. One may postulate that they function to stabilize the heterodimer for presentation on the cell surface in the case of MHC class II molecule; alternatively, they may compete for presentation with antigenic peptides, thereby increasing the threshold for antigenic stimulation. The expression of endogenous antagonists by MHC class II-positive cells in the spleen and thymus in TCR transgenic mice has been demonstrated to result in tolerance by peripheral antagonism rather than by central negative selection mechanisms (10). Several groups have now shown that peptides derived from conserved regions of both class I and II MHC molecules may inhibit the autoreactive response in vitro (11). More significantly, MHC class I–derived peptides have effectively prolonged allograft survival in vivo in several small animal models. Although these peptides have not been as effective as mechanisms targeting co-stimulatory pathways (12), other rationally designed peptides that mimic the putative interaction site of CD4 and the MHC class II molecule have been shown to have significant benefits in animal models of experiment autoimmune encephalomyelitis, allogeneic bone marrow transplantation, and skin transplantation (13,14). Thus, MHC-related peptide-induced antigen-specific unresponsiveness represents a novel form of immunomodulation.

Our initial investigations focused on the influence of a group
of peptides derived from a highly conserved region of the α chain (residues 62 to 77) of three class II MHC molecules (15). All three peptides inhibited the rat MLR independent of responder or stimulator MHC. The most effective of these peptides, HLA-DQA1, was a potent inhibitor of the mouse, rat, and human MLR. In addition, HLA-DQA1 prevented the generation of cytotoxic T lymphocytes (CTL). Inhibition by HLA-DQA1 was shown to be mediated through the induction of apoptosis. We now report on the novel mechanism of action of the nonpolymorphic MHC class II–derived peptide, HLADQA1. Our data provide evidence to suggest that HLA-DQA1 mediates its immunomodulatory effects through induction of apoptosis in APC and T cell hyporesponsiveness. In addition, it is a potent inhibitor of the delayed type hypersensitivity response in vivo by preventing both the initial priming to allogeneic cells and also the response of primed lymphocytes.

**Materials and Methods**

**Peptides**

Peptides derived from conserved regions of class II MHC were synthesized by Global Peptide Services (Fort Collins, CO) using an automated peptide synthesizer, purified by reverse phase HPLC, and shown to be greater than 95% homogenous by analytical reverse HPLC and mass spectroscopy (15). The immunogenic peptide RT1.Dβ2 (20–44), a Wistar Furth (WF) class II MHC peptide, was synthesized by Macromolecular Resources (Fort Collins, CO), purified by reverse phase HPLC, and shown to be greater than 80% homogenous by analytical reverse HPLC and mass spectroscopy (16). Before use, the peptides were dissolved in sterile phosphate-buffered saline at a concentration of 1 mg/ml.

**Antibodies and Reagents**

Antibodies to CD11b, CD19, CD4, CD8, CD95, Annexin-V, and the corresponding isotype controls were purchased from BDPharmingen (San Diego, CA). Recombinant mouse IL-2 and hamster antimese CD28 monoclonal antibody (mAb) were purchased from BD-Pharmingen (San Diego, CA). The caspase inhibitors Z-VAD-Fmk and Boc-D-FMK, propidium iodide, and LPS were purchased from CalBiochem (La Jolla, CA). 7-AAD was purchased from BDPharmingen (San Diego, CA).

**Animals**

Adult male C57BL/6, BALB/c, and BALB/c nude mice (age, 4 to 6 wk) were purchased from the Jackson Laboratories (Bar Harbor, ME).

**Proliferation Assay**

Inbred responder BALB/c mice were primed by immunization subcutaneously in footpads and axillae with 100 μg of the Wistar Furth peptide RT1.Dαβ2 (20–44) in complete Freund adjuvant. Two weeks after immunization, popliteal and axillary lymph nodes were harvested and the lymphocytes isolated as described previously (15). The cells were then washed twice and resuspended into RPMI 1640 medium and suspended at a concentration of 10^6 cells for 30 min on ice and washed once. The lymphocytes were then incubated with either FITC- or PE-conjugated experimental antibodies for 45 min, washed, and resuspended in 50 μl of PBS + 0.5% BSA. Cell incubated with FITC- or PE-conjugated isotype control antibodies served as controls. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

**Isolation of Peritoneal Macrophages**

Naïve BALB/c mice were sacrificed, the peritoneum was exposed under sterile conditions, and 8 ml of ice-cold RPMI 1640 medium was injected into the peritoneal cavity (BioWhitaker Inc., Walkersville, MD). The abdomen was gently agitated, and the fluid slowly withdrawn through the syringe. The cells were washed twice in ice-cold medium and suspended at a concentration of 1 × 10^6. Staining with CD11b demonstrated >90% macrophages. Macrophages (5 × 10^5) were added to 12-well plates and incubated with RT1.Dαβ2 at a final concentration of 50 μg/ml for 2 h, excluding negative control. At this time lymphocytes primed to RT1.Dαβ2 were added alone or with either HLA-DQ A1 (100 μg/ml) or HLADQB1 (100 μg/ml) to the appropriate wells. Cells were harvested at 24 h and assessed for apoptosis.

**Lymphocyte Restimulation**

BALB/c lymphocytes (1 × 10^6/ml) primed to RT1.Dβ2, by immunization were stimulated by RT1.Dβ2 (50 μg/ml) in the presence or absence of either HLA-DQA1 (100 μg/ml) or HLADQB1 peptides (100 μg/ml). In the case of the dose-response experiments, cells were stimulated with RT1.Dβ2 in the presence of increasing doses of HLA-DQA1 or HLADQB1 (1.5 to 100 μg/ml). Negative control wells were set up with culture medium alone. The plates were incubated at 37°C with 5% CO₂ for 72 h with addition of 3H-thymidine (1 μCi/well, NEN Dupont, Boston, MA) for the last 18 h of culture. Cells were then harvested with a Tomtec Harvester 96. Proliferation was assayed by 3H-thymidine uptake. Experimental wells were set up in quadruplicate, and results are expressed as mean counts per minute (CPM ± SEM).

**Quantitation of Apoptosis by Flow Cytometry**

BALB/c lymphocytes (1 × 10^6/ml) primed to RT1.Dβ2, by immunization were stimulated by RT1.Dβ2 (50 μg/ml) in the presence or absence of either HLA-DQA1 (100 μg/ml) or HLADQB1 (100 μg/ml). Cells incubated in medium alone served as negative control. The induction of apoptosis was assessed by cell cycle analysis after propidium iodide uptake as described previously (15). In addition, apoptosis was demonstrated by staining for annexin V-PE in combination with the viability dye 7-AAD. Cells that stained positive for annexin V-PE alone represented apoptotic cells, the combination of positive annexin V-PE and 7-AAD staining represents dead apoptotic cells, and cells positive for 7-AAD alone are necrotic cells. In the case of LPS B cells (1 × 10^6/ml) from BALB/c Nude mice were stimulated with 15 μg/ml of LPS ± HLA-DQA1 (100 μg/ml) or HLADQB1 (100 μg/ml) for 24 h, and apoptosis was assessed by cell cycle analysis after propidium iodide uptake. Cells incubated in medium alone served as negative control.

**Phenotypic Analysis by Flow Cytometry**

Lymphocytes (2.5 × 10^5/ml) primed to RT1.Dαβ2 were stimulated with RT1.Dαβ2 at a concentration of 50 μg/ml in the presence or absence of HLA-DQA1 (100 μg/ml) or HLADQB1 peptides (100 μg/ml) for either 2 or 24 h. At this time, cells were washed twice in PBS + 0.5% BSA. Lymphocytes were incubated with the CD16/CD32 blocking antibody at a concentration of 1 μg/1 × 10^6 cells for 30 min on ice and washed once. The lymphocytes were then incubated with either FITC- or PE-conjugated experimental antibodies for 45 min, washed, and resuspended in 500 μl of PBS + 0.5% BSA. Cell incubated with FITC- or PE-conjugated isotype control antibodies served as controls. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).
abdominal wall with 1/2600 magnification. The whole tissue section (a transverse foot with footpad benzidine (Vector Labs), and counterstained in hematoxylin. Isotype-Elite kit Vector Labs, Burlingame, CA), visualized with Diaminobenzidine (macrophage marker), using the avidin-biotin technique (Vectastain with the antibody of interest. The sections were stained for the specific antigenic determinant of interest. The sections were then washed twice to remove excess antibodies before adding lymphocytes initially stimulated with RT1.Dβ2 ± HLA-DQA1 or HLA-DQ β1. The plates were incubated at 37°C with 5% CO2 for 48 h with addition of 3H-thymidine (1 μCi/well, NEN Dupont, Boston, MA) for the last 18 h of culture. Cells were then harvested with a Tomtec Harvester 96. Proliferation in quadruplicate, and results are expressed as mean CPM ± SEM.

Delayed Type Hypersensitivity Response

C57BL/6 mice (n = 4/group) were injected intradermally in the abdominal wall with 1 × 107 BALB/c splenocytes alone or in the presence of either the inhibitory peptide HLA-DQA1 (200 μg/ml) or the control peptide HLA-DQβ1 (200 μg/ml). Mice were injected subcutaneously in one footpad 5 d later with 1 × 106 irradiated BALB/c spleen cells, and the contralateral foot was injected with PBS as control. Baseline measurements of footpad thickness were taken before injection using a micrometer. After 2 d, the footpads were measured again and the delta footpad thickness compared between experimental groups. In separate experiments C57BL/6 mice were initially primed with 1 × 107 BALB/c splenocytes alone and then subsequently challenged with BALB/c splenocytes alone or in the presence of either the inhibitory peptide HLA-DQA1 (200 μg/ml) or the control peptide HLA-DQβ1 (200 μg/ml). Delta footpad thickness was again compared between experimental groups. Statistical analysis was assessed by ANOVA.

Immunohistology and Histopathology

Footpad samples were collected from three mice in each experimental group. Footpad tissues were embedded in OCT, quick frozen in liquid nitrogen, and kept at −70°C until sectioning. Cryostat sections (6 μm) of footpad were fixed with acetone and then labeled with the antibody of interest. The sections were stained for the specific cell surface markers, CD4 (PharMingen, San Diego CA) and F4/80 (macrophage marker), using the avidin-biotin technique (Vectastain Elite kit Vector Labs, Burlingame, CA), visualized with Diaminobenzidine (Vector Labs), and counterstained in hematoxylin. Isotype-matched Ig and omission of the primary antibody served as negative controls. Each specimen was evaluated at least at three different levels of sectioning. The whole tissue section (a transverse foot with footpad section) was evaluated for a given cellular marker at 100 to 400 magnification.

Results

Inhibition of Proliferation in Murine Lymphocytes

We have previously demonstrated that HLA-DQA1 (62–77) inhibits T cell proliferation in an allele and species nonspecific manner (15). To further study the mechanism of action of this immunomodulatory peptide, we used an antigen-specific mouse model in which BALB/c T cells were primed by immunization to the Wistar Furth rat MHC class II peptide, RT1.Dβ2 (20–44). This model was based on previous studies examining the indirect pathway of xenorecognition, in which we demonstrated that RT1.Dβ2 was immunogenic in BALB/c mice when presented in a self-restricted manner by class II MHC and induced T cell proliferation (16). HLA-DQB1 (62–77), which is derived from a similar region of the HLA-DQ beta chain, has no inhibitory effect and was used as control peptide. We found that when BALB/c lymphocytes primed to RT1.Dβ2 by immunization were stimulated with RT1.Dβ2 in the presence of HLA-DQA1 proliferation was inhibited, with 77.2 ± 2.4% inhibition at 100 μg/ml as compared with 5.9 ± 10.3% inhibition with HLA-DQβ1 (100 μg/ml) (P < 0.0001) (Figure 1A). In addition, HLA-DQA1 inhibits proliferation in a dose-dependent manner (Figure 1B). Thus, HLA-DQA1 is a potent inhibitor of antigen-specific lymphocyte proliferation. Inhibition of proliferation may be mediated by several potential mechanisms, including anergy or deletion. To investigate whether inhibition of proliferation by HLA-DQA1 was mediated by anergy, exogenous rIL-2 (5 ng/ml) was added at the initiation of cell cultures and the proliferative response examined. Although the addition of rIL-2 did increase proliferation in the presence of HLA-DQA1, the inhibitory effect was not completely reversed, with no statistically significant difference demonstrated after the addition of rIL-2 as compared with either RT1.Dβ2 alone or in combination with rIL-2 (Figure 1C). These results suggest that classical IL-2 responsive anergy does not fully account for the unresponsiveness induced by HLA-DQA1 (17). In contrast, the addition of a stimulatory anti-CD28 mAb (2 μg/ml) resulted in complete reversal of the inhibitory effect seen with HLA-DQA1 (Figure 1D).

Induction of Apoptosis in Murine Lymphocytes

We next examined whether inhibition of proliferation of murine lymphocytes caused by HLA-DQA1 was mediated by apoptosis, as shown in previous studies with rat lymphocytes (15). BALB/c lymphocytes primed to RT1.Dβ2 by immunization were stimulated in the presence or absence of either HLA-DQA1 or HLA-DQβ1, and apoptosis determined at 24 h. Cells incubated in medium alone served as negative control. Stimulation of lymphocytes in the presence of HLA-DQA1 resulted in 72.6 ± 77.2% apoptosis compared with 28.9 ± 3.65%, 34.2 ± 3.5%, and 33.4 ± 2.79% in negative control, RT1.Dβ2 alone, and HLA-DQβ1, respectively (P < 0.0001; n = 9). We have demonstrated that HLA-DQA1 had no effect on naïve rat lymphocytes. In contrast, naïve murine lymphocytes underwent apoptosis in the presence of HLA-DQA1, 49.9 ± 5.4% compared with 35.1 ± 3.2% and 34.6 ± 3.2% spontaneous apoptosis with medium and HLA-DQβ1, respectively (n = 4). However, this was significantly less than that seen in lymphocytes activated in the presence of HLA-DQA1 (P = 0.01). We also examined for apoptosis using annexin V-PE/7-AAD staining. We found that as early as 2 h after peptide exposure there was an increased percentage of early apoptotic and apoptotic dead cells with HLA-DQA1 (Figure 2). Early apoptotic and apoptotic dead cells represented 72.6–77.2% apoptosis compared with 28.9 ± 3.65%, 34.2 ± 3.5%, and 33.4 ± 2.79% in negative control, RT1.Dβ2 alone, and HLA-DQβ1, respectively (P < 0.0001; n = 4 experiments). No increase in 7-AAD staining alone was seen; therefore, cells
were not necrotic. Thus, these data suggest that inhibition of proliferation of antigen-specific murine lymphocyte by HLA-DQA1 is mediated through the induction of apoptosis.

Phenotype of Cells Undergoing Apoptosis

To delineate the cell population undergoing apoptosis, primed lymphocytes stimulated with RT1.Dβ2 ± HLA-DQA1 or HLADQB1 in vitro were examined for the percentage CD4+ and CD8+ T cells and B cells (anti-CD19 mAb) at 2 and 24 h. We found that at 2 and 24 h the percentage CD4+ and CD8+ cells was increased with HLA-DQA1 (Figure 3). However, when cell number was taken into consideration, there was no absolute increase in CD4+ or CD8+ cells; thus the increase in percentage represents a reduction in the total number of cells. Staining with anti-CD19 mAb revealed that the reduction in total number of cells was accounted for by a reciprocal decrease in the B cell population. This decrease in B cells was evident as early as 2 h, with a more dramatic decrease by 24 h (Figure 3). In addition, the decrease in B cells demonstrated a dose-response effect of HLA-DQA1, with CD19-positive cell counts of 514.7 ± 317, 657 ± 273, 900 ± 272, 1182 ± 241 at doses of 100 μg/ml, 75 μg/ml, 50 μg/ml, and 25 μg/ml, respectively, as compared with 1666 ± 324 and 2502 ± 1253 for RT1.Dβ2 alone and HLA-DQB1 100 μg, respectively. To

Figure 1. Inhibition of proliferation of murine lymphocytes by HLA-DQA1. (A) BALB/c lymphocytes primed by immunization to the immunogenic xeno-MHC peptide RT1.Dβ2 were stimulated \textit{in vitro} in the presence or absence of HLA-DQA1 or control peptide HLA-DQB1. Proliferation was determined by 3H thymidine uptake after 72 h and is expressed as counts per minute (CPM ± SEM). Results are shown as percent inhibition for (A) HLA-DQA1 and HLA-DQB1 at 100 μg/ml compared with control groups (*P < 0.0001 compared with other experimental groups; n = 11) and (B) dose response for HLA-DQA1 and HLA-DQB1 (n = 3). In addition, (C) r-IL2 or (D) a stimulatory anti-CD28 antibody were added to cultures. Proliferation was determined by 3H-thymidine uptake after 72 h and is expressed as CPM ± SEM (n = 4). (C) Proliferation of cells stimulated in the presence of HLA-DQA1 ± IL-2 was not significantly different from RT1.Dβ2 alone or RT1.Dβ2 + rIL-2 (*P = NS). (D) Proliferation of cells stimulated in the presence of HLA-DQA1 ± anti-CD28 antibody was significantly different (*P = 0.0005). Proliferation was not statistically different between RT1.Dβ2 + anti-CD28 antibody and HLA-DQA1+ anti-CD28 antibody (**P = NS).
determine whether the decrease in B cells occurred as a result of apoptosis and therefore accounted for the apoptosis seen with HLA-DQA1, cells were dual-stained with anti-CD19-FITC and anti-annexin V-PE antibodies. An increase in annexin V-PE binding by B cells was observed, with 31.2 ± 3% of cells in the HLA-DQA1 group staining positive for both CD19 and annexin V at 2 h compared with 17.3 ± 2%, 15 ± 1%, and 18.4 ± 2% for medium, RT1.Dβ2 alone, and HLA-DQB1, respectively (P < 0.0038) (Figure 4). The increase in annexin-V binding by B cells increased over time at 15.8 ± 0.13% at 30 min to 23.9 ± 0.14% at 1 h, significantly higher than that seen in control groups (P = 0.0007). This increase in annexin-V by B cells at 30 min and 1 h occurred in the absence of any significant change in the number of B cells between groups. Thus, B cell apoptosis is initiated very early after stimulation of cells in culture in the presence of HLA-DQA1. Dual CD19 and annexin-V staining was not seen at 24 h because B cells were almost completely depleted by this time. Therefore, stimulation of lymphocytes in the presence of HLA-DQA1 induces apoptosis in B cells. To examine whether apoptosis also occurs in other APC, we used peritoneal macrophages pulsed with RT1.Dβ2 to stimulate RT1.Dβ2-primed lymphocytes. We found that macrophages (CD11b) were also reduced considerably by HLA-DQA1 at 24 h, 19.9 ± 1.5% compared with 66.5 ± 2.9%, 61.3 ± 6.5%, and 62.2 ± 9% for media, RT1.Dβ2 alone, and HLADQB1, respectively (P < 0.01). These data suggest that HLA-DQA1 mediates its inhibitory effect through the induction of apoptosis in APC and are consistent with the lack of effect of HLA-DQA1 on APC-independent T cell activation (15).

T Cell–Independent Induction of Apoptosis in APC

We next determined whether the induction of apoptosis in activated APC required the interaction of the T cell and APC or whether T cell–independent stimulation of APC may also be affected. BALB/c nude mice B cells were stimulated with LPS in the presence or absence of HLA-DQA1 or HLA-DQB1 for 24 h and CD19 staining performed. B cells were decreased to 63.3 ± 3.5% by HLA-DQA1 compared with 88.9 ± 2.2% and 88.4 ± 3.1% for LPS and LPS + HLA-DQB1, respectively (P < 0.0001) (n = 5). These data suggest that induction of apoptosis in APC by HLA-DQA1 does not require T cell–
mediated activation. However, the decrease in B cells seen at 24 h when stimulated in a T cell–dependent manner is significantly greater than that seen after stimulation with LPS (P < 0.0001). These data suggest that activation of APC increases apoptosis induced by HLA-DQA1 and the effect is greater after a TCR MHC interaction.

**Induction of Apoptosis via a Nonclassical Pathway**

Apoptosis is morphologically and biochemically distinct from necrosis, and it consists of three major components: the caspases; the Bcl-2 family; and the cell surface receptors such as Fas and TNFR (18). We examined the expression of several members of each of these families by RPA using the Pharmingen mAPO-1, mAPO-2, and mAPO-3 kits (data not shown). We found that there was no change in the level of expression of members of the Bcl-2 family or cell surface receptors among experimental groups at 2, 6, and 24 h. In particular, Bax or Bak, which are proapoptotic, were not increased, and Bcl-xL and Bcl-2, which protect against apoptosis, were not decreased. In addition, there was no increase in expression of Fas or TNFR and their related proteins in cells treated with HLA-DQA1. These data suggest that HLA-DQA1 apoptosis is independent of Fas or TNFR signaling. Furthermore, the rapid timing of the apoptosis mitigates against Fas and TNFR-mediated cell death, because apoptosis secondary to these pathways usually occurs over a longer time period (19). Cell surface expression of Fas is not completely regulated at the RNA level because transport of Fas stored in the Golgi apparatus can occur (20). We, therefore, examined cell surface expression of Fas by B cells using flow cytometry and dual staining for CD19-FITC and CD95-PE. No increase in Fas expression by B cells was observed at 2 h (n = 3) (data not shown). These data lend further support to our findings that the induction of apoptosis in B cells is Fas independent.

There is now growing evidence that caspase-independent pathways may mediate apoptosis (21). We found no difference between experimental groups in the level of expression of caspases as determined by RPA, there was particularly no difference in critical induction or effector caspases such as 1, 8, 3, or 9 at 2, 6, and 24 h (n = 3; data not shown). We further investigated whether apoptosis induced by HLA-DQA1 was mediated by caspases by preincubating primed murine lymphocytes with one of two caspase inhibitors, zVAD-fmk or BOC-D-fmk, for 2 h before stimulation with RT1.Dβ2 alone or in the presence of HLA-DQA1 or HLA-DQB1. Both of these inhibitors successfully prevented apoptosis in controls in which apoptosis was induced by stimulation with RT1.Dβ2 alone or when treated with mFas antibody. However, no inhibition was seen with either reagent in lymphocytes stimulated in the presence of HLA-DQA1 (Figure 5, A, B, and C). Taken together, these data strongly suggest that nonpolymorphic MHC class II peptides mediate apoptosis through a nonclassical caspase-independent mechanism.

**Lymphocyte Restimulation**

To determine whether the responsiveness of T lymphocytes stimulated in the presence of HLA-DQA1 were affected, we examined the proliferative response of these lymphocytes upon restimulation with APC pulsed with RT1.Dβ2 in the absence of HLA-DQA1. We found that T cells initially stimulated in the presence of HLA-DQA1 had a diminished proliferative response when rechallenged with RT1.Dβ2 alone (Figure 6). The proliferative response of T lymphocytes from the HLA-DQA1 experimental group was 11,435 ± 4412 (CPM ± SEM) as compared with 28,234 ± 5887 and 21612 ± 3997 for the lymphocytes initially stimulated with RT1.Dβ2 alone (P = 0.05) and RT1.Dβ2 + HLA-DQB1 (P = NS), respectively (n = 4). Thus, although there is evidence of proliferation, it is markedly diminished compared with controls. These data would suggest that in addition to inducing apoptosis in APC, HLA-DQA1 renders T cells hyporesponsive and provides an explanation for the partial response seen with the addition of rIL-2.

**HLA-DQA1 Prevents the Priming and Response of Allogeneic T Cells In Vivo**

Using a delayed type hypersensitivity (DTH) model we investigated the ability of nonpolymorphic MHC class II peptides to inhibit the alloimmune response in vivo. C57BL/6 mice were injected intradermally with BALB/c splenocytes alone or in combination with either HLA-DQA1 or HLA-DQB1 (200 μg/ml), and the DTH response was subsequently determined. We found that there was a significant reduction in the delta footpad thickness (ΔDTH) in animals co-injected with HLA-DQA1, with a 73.6 ± 12% reduction in the ΔDTH compared with animals injected with cells alone (Figure 7A). No statis-

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*Figure 3. Phenotypic analysis. To determine the cell population undergoing apoptosis, BALB/c lymphocytes primed to RT1.Dβ2 were stimulated in vitro in the presence or absence of HLA-DQA1 or control peptide HLA-DQB1 for 2 or 24 h. At these time points, cells from each experimental group were stained for CD4, CD8, and CD19 and analyzed by flow cytometry. This graph represents the percentage of the cell populations comprised of CD4+ T cells, CD8+ T cells, and B cells for medium, RT1.Dβ2, HLA-DQA1 and HLA-DQB1 at 2 and 24 h for four combined experiments. * P < 0.0001, ** P = 0.0028, # P = 0.044.*
tical difference was seen with HLA-DQB1. To determine whether HLA-DQA1 could prevent the development of the DTH response once T cells have been primed, animals were initially injected with allogeneic splenocytes alone and then challenged on day 5 with cells alone or in combination with either HLA-DQA1 or HLA-DQB1. HLA-DQA1 again prevented the development of the DTH response with a 73.8 ± 24.6% reduction in the DTH (Figure 7B). Of note, the dose of HLA-DQA1 used in vivo (200 μg/ml) is comparatively less than that used in vitro (100 μg/ml) because a thousand-fold more cells are used in vivo. No inhibition of the DTH response was seen when 20 μg/ml of HLA-DQA1 was administered in conjunction with cells at the time of priming or subsequently with the injection of cells in the footpad. To determine whether co-administration of cells and peptide were required to inhibit the DTH response, HLA-DQA1 was injected at the same time as allogeneic cells but separately on the contralateral side of the abdomen. We found that HLA-DQA1 still inhibited the DTH by 87.8 ± 8% (Figure 7C). Immunohistology was performed for CD4 and F4/80 (macrophage marker) on the footpads taken at the time of measurement of the DTH response (Figures 8 and 9). Animals in which HLA-DQA1 was administered at the time of priming, either together with cells or at a different site, demonstrated a marked reduction of CD4⁺ T cell and macrophage infiltrates as compared with cells alone and cells plus control peptide, HLA-DQB1. Co-administration of HLA-DQA1 at the time of injection of the footpad with cells resulted in a reduction in cellular infiltrates. However, in the case of CD4⁺ T cells, this was not as marked as that seen when the inhibitory peptide was administered at the time of priming, suggesting that there is recruitment of primed CD4⁺ T cells but a decreased response. Flow cytometry of the draining lymph nodes in each of the experimental groups demonstrated no appreciable difference in annexin V staining or cell phenotype as determined by staining for CD4, CD8, or CD19 (data not shown). Taken together, these data demonstrate that HLA-DQ1 prevents both the priming of allogeneic T cells and also the response of primed allogeneic T cells in vivo.

Discussion
We have previously demonstrated that a group of peptides derived from a region of MHC class II that is conserved between alleles and species effectively inhibit T cell proliferation in an allele and species-nonspecific manner (15). In this
article, we further investigate the mechanisms mediating their effects using HLA-DQA1. We provide evidence that inhibition of proliferation of murine lymphocytes by nonpolymorphic MHC class II peptides, specifically HLA-DQA1, is mediated through the combination of the induction of apoptosis in APC and T cell hyporesponsiveness. Two broadly inhibiting caspase inhibitors did not prevent the apoptosis, implying that HLA-DQA1 induces apoptosis via a nonclassical apoptotic pathway. Caspase-independent cell death has been demonstrated after the ligation of receptors including CD47, CD45, CD99, class I MHC, and class II MHC (21–25), or apoptosis induced by receptor-independent means such as irradiation or chemotherapeutics (26). It is possible, however, for caspase inhibitors to prevent some but not all the biochemical and morphologic characteristics of apoptosis (26,27). Therefore, we cannot rule out that apoptosis induced by HLA-DQA1 may be mediated by alterations in the mitochondrial membrane and that cell death still occurs in the presence of caspase inhibitors.

Both MHC class I and class II molecules have been shown to have immunosuppressive effects in vitro; however, the biologic significance of this in vivo has yet to be established. Zavazava and Kronke (28) have demonstrated that soluble MHC class I can induce apoptosis in alloreactive CD8+ T cells. Anti-HLA class I antibodies to both the α2 and α3 domains have all been shown to induce T cell apoptosis in a fas-independent manner (25,29), whereas antibodies to the α1 domain may trigger apoptosis in activated T cells and B cells (30,31). In addition, apoptosis in B cells, macrophages, and dendritic cells have all been shown to occur after MHC class II signaling through either caspase-dependent or -independent mechanisms (21,32). The lack of effect seen when HLA-DQA1 is preincubated with APC (15) and the rapidity with which it acts argue against the hypothesis that HLA-DQA1 binds within

![Figure 5. Apoptosis induced by HLA-DQA1 is unaffected by caspase inhibitors. BALB/c lymphocytes primed to RT1.Dυ2 were incubated with or without the two caspase inhibitors, (a) Boc-D-fmk (n = 4) or (B) Z-VAD-fmk (n = 4) for 2 h before stimulation with RT1.Dυ2 alone or in the presence of either HLA-DAQ1 or HLA-DQB1. Apoptosis was quantitated at 24 h by propidium iodide uptake and is expressed as percent apoptosis ± SEM for four experiments. Induction of apoptosis in unstimulated lymphocytes by monoclonal Fas antibody served as positive control. (C) Facs analysis at 24 h demonstrating the effect of Boc-D-fmk and Z-VAD-fmk on apoptosis in lymphocytes stimulated in the presence of HLA-DQA1 or unstimulated cells incubated with mFas Ab. This experiment is representative of four experiments.](image)
the MHC-peptide binding groove, thereby altering the T cell response. In a recent publication describing the crystalline structure of a TCR in complex with a peptide/MHC class II complex, residues 60 and 61 of the α1 helix were recognized by the T cell receptor (33). These residues are adjacent to those from which HLA-DQA1 (62–77) is derived. It is therefore possible that HLA-DQA1 binds to MHC class II at an alternative site critical for TCR–MHC interaction. It may therefore be postulated that HLA-DQA1 disrupts the MHC-TCR interaction, resulting in the induction of apoptosis in APC and altering T cell signaling events rendering the T cell unresponsive. We have previously shown that HLA-DQA1 has no effect on rat naïve lymphocytes. In these current studies, we show apoptosis in naïve murine lymphocytes; however, the degree of apoptosis is dramatically enhanced after presentation of antigen by APC to primed T cells. This increase in apoptosis in APC seen when interacting with T cells would imply that the effect is an active rather than a passive process and that the lack of T cell proliferation does not occur because of the random depletion of APC. The potential requirement for MHC class II molecules for the effects of the nonpolymorphic peptides was initially suggested by data demonstrating that preformed rat CTL are not inhibited by HLA-DQA1 (15). In addition, the ability of HLA-DQA1 to prevent superantigen-mediated proliferation but not that due to mitogen suggests that HLA-DQA1 may disrupt the T cell–APC interaction. We demonstrate that HLA-DQA1 induced apoptosis after MHC class II–independent stimulation of APC. Although it has been previously demonstrated that stimulation of B cells with LPS does not prevent MHC class II–induced apoptosis (34), induction of apoptosis by HLA-DQA1 after LPS stimulation may also suggest that its effect is independent of the MHC class II molecule. Abrogation of the inhibitory effect of HLA-DQA1 by anti-CD28 suggests that T cell–APC interactions are taking place. Additional co-stimulation may overcome weakening in the TCR–MHC/peptide interaction and account for the abrogation of the inhibitory effect by anti-CD28 (35). Alternatively, HLA-DQA1 may bind to a costimulatory molecule thereby interfering with the second signal. This would be consistent with the increased effect of HLA-DQA1 on stimulation and the synergistic effect of CTLA4Ig on the inhibition of proliferation and cytokine production and the T cell hyporesponsiveness induced by HLA-DQA1 (15). However, we cannot rule out the possibility that the effects seen with anti-CD28 occur as a result of increased stimulation of T cells by a small amount of remaining APC, for instance dendritic cells.

This is the first demonstration that MHC class II–derived peptides prevent the immune response in vivo and that this effect is mediated in part through the novel mechanism of deletion of APC. HLA-DQA1 prevents both the priming of
allogeneic T cells and also the response of previously primed T cells. In addition, the inhibitory effect in vivo occurs if HLA-DQA1 is administered separately from cells, most likely by systemic absorption. Thus, targeting APC combined with T cell hyporesponsiveness is an effective means of preventing the immune response in vivo. No currently used immunosuppressant has been shown to specifically target APC in allorecognition, the depletion of APC may be particularly useful in the direct pathway, in which donor APC are the target for the immune response. In addition, donor APC naturally decline over the first few months; hence the deletion of APC would not be indefinitely required (36,37). HLA-DQA1 has been shown to inhibit the indirect pathway of allorecognition in vitro. Deletion of all APC in this pathway may represent a formidable challenge because this is likely to be an ongoing process mediated by the presentation of allopeptides by MHC on host APC. However, early intervention may prevent amplification of the response due to the release of cryptic epitopes in a process called epitope spreading (38). The importance of antibodies in many stages of the rejection process is being increasingly recognized (39,40). Furthermore, patients with a high percentage of antibodies directed toward HLA wait longer for transplantation; once transplanted have a decreased long-term graft survival (41,42). Specific therapies that target B cells, such as HLA-DQA1, may represent a strategy for the treatment of the highly sensitized patients pretransplant and peritransplant. HLA-DQA1 may have potential applications in other disease processes in which antibody production plays a significant role, such as xenotransplantation (43) and autoimmunity. The effect of HLA-DQA1 would not be limited to the deletion of B cells and the prevention of antibody production, but also the prevention of T cell responses, because we have shown that HLA-DQA1 renders T cell hyporesponsive. Importantly, HLA-DQA1 prevents the response of primed T cells because, unlike transplantation, the initiation of the immune response in autoimmunity cannot be anticipated. Although these potential applications sound promising, there are definite problems with the application of peptide-based strategies in vivo (8). Peptides may have a low oral bioavailability and are rapidly broken down by plasma proteases, thus making it

Figure 8. HLA-DQA1 reduces the cellular infiltration in response to allogeneic cells. Immunohistology was performed for CD4 on footpads taken after the measurement of the DTH response in each experimental group: (a) cells alone; (b) subcutaneous injection of HLA-DQA1 in conjunction with allogeneic cells in abdominal wall; (c) subcutaneous injection of HLA-DQA1 in conjunction with allogeneic cells in footpad; (d) subcutaneous injection of HLA-DQB1 in conjunction with allogeneic cells in abdominal wall; and (e) subcutaneous injection of HLA-DQA1 in abdominal wall at a site separate from allogeneic cells. The degree of cellular infiltrates was reflected by the Δ DTH in each group. Slides shown are representative of three animals in each experimental group.
difficult to establish a significant plasma half-life. However, data using MHC class I peptide in vivo in human renal allograft recipients suggests that this may not necessarily be the case (44). Alternatively, it may be possible to circumvent the difficulty of plasma proteases by fusion of the active peptide to a carrier molecule or synthesis of variants resistant to enzymatic degradation. Gene therapy represents another potential solution, because gene transfer of a immunomodulatory MHC class I peptide successfully prolonged cardiac allograft survival in mice (45).

In summary, these data demonstrate that a synthetic peptide derived from a conserved region of MHC class II can modulate the immune response in vitro and in vivo through the induction of apoptosis in APC via a caspase-independent mechanism and also T cell hyporesponsiveness. HLA-DQA1 prevents both the priming of alloge neic T cells, and also the response of T cells once primed, in vivo. This is the first demonstration of the ability of MHC class II–derived peptides to inhibit the immune response in vivo. These data suggest that the deletion of APC represents a novel method and effective form of immunotherapy when combined with an altered T cell response. Further elucidation of the exact binding site of this immunomodulatory peptide may allow the design of more potent and specifically acting derivatives for in vivo use in experimental animals and humans.

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