Analysis of the Role of Variation of Major Histocompatibility Complex Class II Expression on Nonobese Diabetic (NOD) Peripheral T Cell Response

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Summary

The current paradigm of major histocompatibility complex (MHC) and disease association suggests that efficient binding of autoantigens by disease-associated MHC molecules leads to a T cell–mediated immune response and resultant autoimmune sequelae. The data presented below offer a different model for this association of MHC with autoimmune diabetes. We used several mouse lines expressing different levels of I-A^{g7} and I-A^{k} on the nonobese diabetic (NOD) background to evaluate the role of MHC class II in the previously described NOD T cell autoproliferation. The ratio of I-A^{g7} to I-A^{k} expression correlated with the peripheral T cell autoproliferative phenotype in the mice studied. T cells from the NOD, [NOD × NOD.I-A^{null}]F1, and NOD I-A^{k} transgenic mice demonstrated autoproliferative responses (after priming with self-peptides), whereas the NOD.H2^{h4} (containing I-A^{k}) congenic and [NOD × NOD.H2^{h4} congenic]F1 mice did not. Analysis of $CD4^{+}$ NOD I-A^{k} transgenic primed lymph node cells showed that autoreactive $CD4^{+}$ T cells in the NOD I-A^{k} transgenic mice were restricted exclusively by I-A^{g7}. Considered in the context of the avidity theory of T cell activation and selection, the reported poor peptide binding capacity of NOD I-A^{g7} suggested a new hypothesis to explain the effects of MHC class II expression on the peripheral autoimmune repertoire in NOD mice. This new explanation suggests that the association of MHC with diabetes results from "altered" thymic selection in which high affinity self-reactive (potentially autoreactive) T cells escape negative selection. This model offers an explanation for the requirement of homozygous MHC class II expression in NOD mice (and in humans) in susceptibility to insulin-dependent diabetes mellitus.

Key words: nonobese diabetic • insulin-dependent diabetes mellitus • thymic selection • T cell receptor repertoire • major histocompatibility complex and disease

Most human autoimmune diseases, such as type I IDDM and rheumatoid arthritis, are polygenic in nature (1), making it important to develop animal models of disease-locus–related immune function in polygenic diseases. The nonobese diabetic (NOD)[1] mouse is a model of complex, polygenic disease, in which multiple alleles interact to produce an autoimmune phenotype (2). Of the 18 insulin-dependent diabetes (Idd) loci identified to date in NOD mice, the strongest contribution to disease is from Idd1, which maps to the MHC region and consists of the MHC class II molecule (I-A^{g7}; reference 2) and possibly a second gene product (3). The MHC class II molecules are central to the autoimmune processes, but their exact role has remained controversial. Homozygous I-A^{g7} expression has been shown to be necessary, although not sufficient, for the development of diabetes. In one report, transgenic expression of IL-10, in combination with I-A^{g7} homozygosity but in the absence of other NOD alleles, was sufficient to induce diabetes (4). The association between MHC haplotype and autoimmune diseases in general has been established for over two decades (5). Although several mechanisms to explain this association have been proposed, the actual mechanism(s) remains unclear (6). Genetic linkage association with diabetes in humans and mice is particularly striking in the requirement for MHC class II homozygosity. Approximately 96% of North American type I diabetics are homozygous for expression of the DQβ 57 non-ASP haplotype (7, 8). Similarly, NOD mice are homozygous for a

[1]*Abbreviations used in this paper:* HEL, hen egg lysozyme; Idd, insulin-dependent diabetes; MM, mouse myoglobin; NOD, nonobese diabetic; PI, propidium iodide; SWM, sperm whale myoglobin.

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non-ASP amino acid, serine, at position 57 of the murine counterpart of DQβ, I-A^{g7} (9). NOD F1 mice, heterozygous for non-ASP I-Aβ 57, demonstrate at most only ~3% incidence of diabetes, whereas >95% of diabetic NOD F2 mice (from various breedings) are H-2^{g7} homozygotes (10–12). This requirement for MHC class II homozygosity must be explained in any model of the role of I-A^{g7} in the pathogenesis of IDDM. The association of an MHC class II molecule (requiring a non-ASP amino acid at β chain position 57) with autoimmune diabetes has been attributed previously to high affinity binding of "diabetogenic" peptides by the MHC molecules (13, 14). However, it is unclear why a twofold decrease in the cell surface expression of MHC class II (from homozygous to heterozygous) results in a ≥30-fold decrease in disease incidence if the non-ASP MHC class II molecule functions as a "good" peptide binder.

We previously reported that immunization of NOD mice with self-peptides in CFA disrupted self-tolerance, allowing $CD4^+$ MHC class II–restricted T cells to recognize and proliferate in response to endogenously processed and presented self-peptides on APCs from naive mice (referred to as "autoproliferation") (15). We report here that quantitative variation of expression of the NOD MHC class II, I-A^{g7}, in relation to a second class II, I-A^k (on a genetic background in which all the other NOD Idd loci except Idd1 are held constant), has a profound effect on the peripheral $CD4^+$ T cell autoimmune repertoire. The analysis of peripheral T cell responses to self-peptides in the different mice (autoproliferation), combined with the demonstration of quantitative differences in the expression of MHC class II gene products, supports the idea of defective thymic selection as a basis for the autoreactive peripheral T cell repertoire in NOD mice. The report that the NOD I-A^{g7} molecule is a poor peptide binder (16), when considered in the context of an avidity theory of T cell thymic selection, suggests a unifying explanation for the NOD phenotype and provides a rationale for the requirement of MHC class II homozygosity in autoimmune diabetes.

Materials and Methods

Mice. The following mice were bred and housed in the Stanford Medical School Department of Comparative Medicine (DCM) under specific pathogen-free conditions: NOD.$H2^{h4}$ N6F16 congenic (reference 17; hereafter designated NOD.I-A^k), B10.NOD-$H2^{g7}$*Idd10* N7F16 congenic (reference 18; hereafter designated B10.$H2^{g7}$) (both gifts of Drs. Linda Wicker and Larry Peterson, Merck and Co., Inc., Whitehouse Station, NJ); [NOD × NOD.I-A^{null}]F1 (−/g7; gift of Ms. Ann Herman, Stanford University School of Medicine); and NOD I-A^k transgenic (gift of Dr. Robyn Slattery, DNAX, Palo Alto, CA). Mice were used between the ages of 6 and 12 wk (prediabetic). NOD and [NOD × NOD.I-A^k]F1 mice were bred and housed in the DCM.

Antigen Proliferation Assays. Peptides mouse myoglobin (MM) 69–78, and MM110–121, sperm whale myoglobin (SWM) 110–121, hen egg lysozyme (HEL) 46–61, and TCR Vβ8.2 38–60 were prepared and HPLC-purified by either the Protein and Nucleic Acid Facility, Beckman Center, Stanford University, or by Dr. Jonathan Rothbard, Stanford University. Mice were immunized intradermally at the base of the tail with an emulsion of either 5× CFA (IFA plus 10 mg/ml of heat-killed *Mycobacterium tuberculosis*, H37RA; Difco, Detroit, MI) alone, or 100 μg of peptide suspended 1:1 in Dulbecco's PBS and mixed with an equal volume of CFA. The mixtures were vortexed for 45 s, then emulsified via sonification before use. 6–14 d after immunization, draining inguinal lymph node cells were removed and single cell suspensions were prepared. 5×10^5 cells were incubated in 96-well flat-bottomed plates in either T cell media alone or with titrated doses of antigen or PPD. T cell media consisted of RPMI 1640 supplemented with 2 mM l-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 10 mM Hepes Buffer (GIBCO BRL, Gaithersburg, MD), 50 mM 2-ME (Sigma Chemical Co., St. Louis MO), and 0.5% normal mouse serum. After 72 h of culture (at 37°C, 6% CO_2), cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested 18 h later. For use as naive APCs, peripheral lymph nodes from naive NOD, NOD.I-A^k, and [NOD × NOD.I-A^k]F1 mice were obtained and single cell suspensions were made and irradiated with 3,300 rads.

Minimacs Purification of CD4 Cells. Single cell suspensions of freshly isolated lymph node cells were counted and incubated with anti-CD4 magnetic microbeads (10 μl beads per 10^7 cells; Miltenyi Biotec, Auburn, CA) for 15 min at 4°C, washed, and purified by passing through magnetic flow columns. The $CD4^+$ cells were counted and 3×10^5 cells were plated with 10^6 naive lymph node cells. After 72 h of culture, the cells were pulsed and counted as above.

FACS Analysis of Peripheral Lymph Node Cells. For analysis of peripheral B cell MHC class II expression, single cell suspensions of peripheral lymph node cells were obtained from naive mice, counted, and incubated with optimal concentrations of anti-B220 FITC alone or in combination with anti-CD3 FITC and anti-Mac-1 FITC (PharMingen, San Diego, CA) and 39J biotin (anti-I-Aα^k) or 10.3.6 biotin (anti-I-Aβ^{g7} and Aβ^k) (PharMingen) in FACS buffer (Dulbecco's PBS plus 2% FCS) for 15 min at 4°C. The cells were then washed and stained in a second step with Streptavidin PE (Caltag Labs., Burlingame, CA) for 15 min at 4°C. The cells were washed and resuspended for FACS® analysis in FACS buffer with propidium iodide (PI). The data was analyzed using the Herzenberg Desk facility plus Flowjo (Tree Star, Inc., San Carlos, CA) on a Power Macintosh. All $B220^+PI^-$ cells were gated for display of MHC class II histograms.

FACS® Analysis of Thymic Cells. For analysis of thymic dendritic cells, thymi were dissected, placed into a solution of 0.5 mg/ml Collagenase D (Boehringer Mannheim, Indianapolis, IN) in Dulbecco's PBS/Hepes, and injected with the Collagenase solution. The thymi were then diced using forceps and scissors, and incubated in the collagenase solution for 45–60 min at 37°C. The thymi were prepared in single cell suspensions by crushing with a syringe and passing through a plastic mesh cell strainer. The cells were washed with tissue culture medium (TCM)/10% FCS, pelleted, resuspended in 20 ml TCM/10% FCS, and placed into culture at 37°C for 2 h. In some experiments the adherent/nonadherent populations were separated at this point, followed by culture overnight of the adherent population and subsequent splitting of nonadherent/adherent cell populations; in other experiments the cells were directly fractionated. In both cases, the cells were pelleted, counted, and stained with anti-CD11c Minimacs magnetic beads (Miltenyi Biotec) at 10 μl per 10^7 cells for 15 min at 4°C. The cells were then passed through a Minimacs column for positive selection. The positively selected cells were stained with anti-CD11c PE (PharMingen) and either AMS 32.1 FITC (PharMingen) or 39J biotin at optimal concentration for 15 min at 4°C.

Control staining with anti-CD4 PE and anti-CD8 FITC (Caltag Labs.) was also performed to identify thymocytes. The cells were washed and the 39J bound cells were stained in a second step with Streptavidin-FITC (Caltag Labs.). The cells were washed and suspended in FACS buffer with PI for analysis by flow cytometry. The data were analyzed using Flowjo with the following parameters: PI^- cells were gated out, and large cells were analyzed for CD11c and class II expression. The CD11c MHC class II high cell population was gated identically across samples for display of MHC expression. Statistical analysis was performed on Excel (Microsoft Corp., Redmond, WA).

Results

After Immunization with Self-peptide, NOD Mice Demonstrate an Autoproliferative Response, Whereas NOD.I-A^k Mice Do Not. Our previous results showed that NOD mice responded to immunization with self-peptides in CFA by developing autoproliferative T cells in the draining lymph node, i.e., primed $CD4^+$ T cells that proliferated in response to naive APCs expressing the endogenously processed and presented self-antigens used for priming (15). We first explored the role of MHC in the autoproliferative response by using NOD and NOD.I-A^k mice (17). After immunization with a self-peptide (TCR Vβ8.2 amino acids residues 38–60), NOD but not NOD.I-A^k mice exhibited autoproliferation (Fig. 1 *A*). NOD.I-A^k mice also showed no autoproliferative response after immunization with several other self-peptides including MM110–121 and MM69–78 (data not shown), which have previously been demonstrated to induce autoproliferation in NOD mice (15). These data demonstrated that introgression of a portion of a non-NOD MHC onto a background of NOD genes either prevented the autoproliferative response, or, conversely that the NOD background genes, in the absence of the NOD MHC, were insufficient for an autoproliferative response.

NOD I-A^k Transgenic Mice Demonstrate an Autoproliferative Response, Whereas [NOD × NOD.I-A^k]F1 Mice Do Not. The loss of an autoproliferative phenotype in the NOD.I-A^k mice could represent a dominant effect of I-A^k expression. To examine this, we bred [NOD × NOD.I-A^k]F1 mice and immunized them with the same set of self-peptides. The [NOD × NOD.I-A^k]F1 mice failed to demonstrate autoproliferation after immunization with the set of self-peptides previously shown to induce autoproliferative responses in NOD mice (reference 15; Fig. 1 *B*), responding in each case in a manner similar to the parental NOD.I-A^k mice. These results were consistent with a dominant effect of I-A^k. [NOD × NOD.I-A^k]F1 mice express I-A^{g7} and I-A^k at heterozygous levels on a NOD background. To further characterize the possible effect of I-A^k expression in the presence of I-A^{g7}, we studied autoproliferation in NOD I-A^k transgenic mice (19). The NOD I-A^k transgenic mice (females) develop diabetes (at reduced levels, ~20–40% incidence compared with female NOD mice ~80%) and their T cells can transfer diabetes to NOD/scid mice (20). The expression of I-A^k as a transgene, in the presence of I-A^{g7}, did not inactivate the autoproliferative response (Fig. 1 *B*). These data were originally difficult to interpret, since both the NOD I-A^k transgenic mice and the [NOD × NOD.I-A^k]F1 mice express I-A^{g7} and I-A^k on a NOD background. Thus, a simple model of a domi-

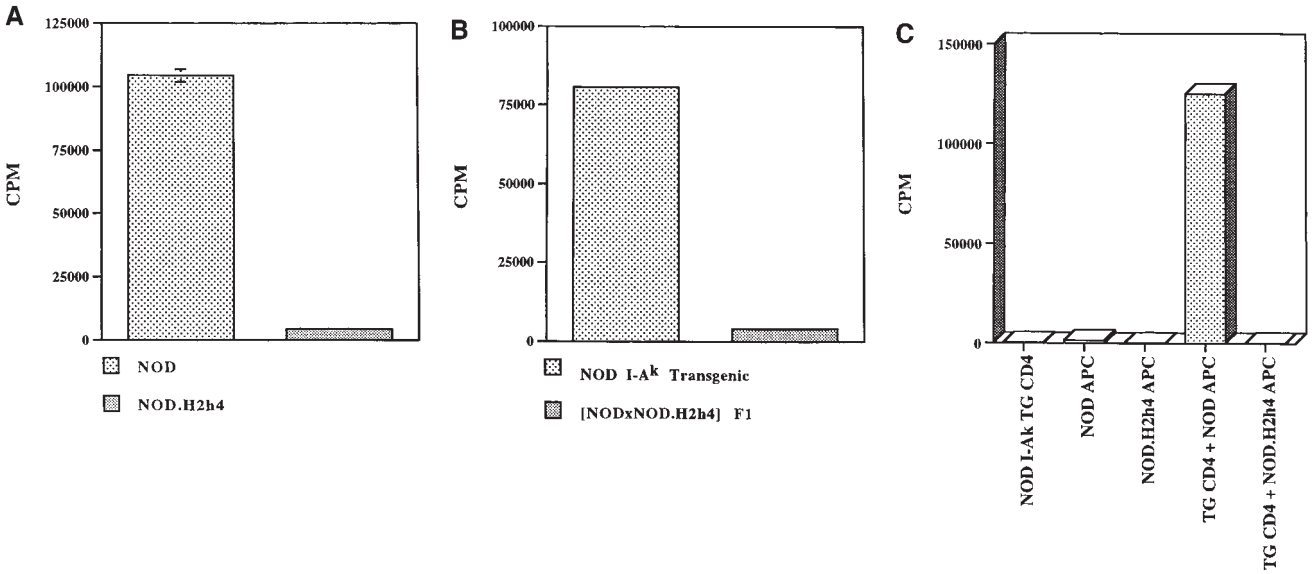


Figure 1. The effect of varying MHC on the autoproliferative response. (*A*) NOD and NOD.I-A^k mice were immunized with self-peptide (TCR) in CFA; 8 d later draining lymph node responses were analyzed in media alone for autoproliferation. One representative experiment of at least four is shown. (*B*) NOD I-A^k transgenic and [NOD × NOD.I-A^k]F1 mice were immunized with a self-peptide (TCR) in CFA; 8 d later the draining lymph node cells were cultured and analyzed for response in media alone. One representative experiment of four is shown. (*C*) NOD I-A^k transgenic mice were immunized as in *A* and *B*. The $CD4^+$ cells from primed lymph nodes were purified by CD4 Minimacs bead columns, then cultured with naive NOD or NOD.I-A^k lymph nodes as APCs in media alone. The transgenic $CD4^+$ cells autoproliferated only in response to NOD APCs. One representative experiment of four is shown.

nant I-A^k effect could not explain the loss of the autoproliferative phenotype in the F1 compared with the transgenic mice.

The Autoproliferative Response in NOD I-A^k Transgenic Mice Is Restricted Solely by I-A^{g7}. If the peripheral T cell repertoire selection in NOD I-A^k transgenic mice was normal on I-A^k but defective on the I-A^{g7} gene product, the autoproliferating T cells from the NOD I-A^k transgenic mice would recognize self-peptide only with I-A^{g7} restriction, not when presented by I-A^k APCs. To test this hypothesis, we purified the $CD4^+$ T cells from the draining lymph nodes of NOD I-A^k transgenic mice immunized with self-TCR peptide in CFA and cultured them with either NOD or NOD.I-A^k lymph node cells from naive mice as APCs. Purified $CD4^+$ T cells from NOD I-A^k transgenic mice autoproliferated in response to the APCs from naive NOD mice (recognizing endogenously processed and presented self-antigen) but showed no response to the APCs from naive NOD.I-A^k mice (Fig. 1 *C*). Thus, the NOD I-A^k transgenic T cell autoproliferative response was entirely I-A^{g7} restricted.

[NOD × NOD.I-A^{null}]F1 mice Show an Autoproliferative Response, whereas [NOD × NOD.I-A^k]F1 Mice Show no Response to Immunization with Self-peptide MM110–121. It remained possible that the lack of an I-A^{g7}–restricted autoproliferative response to immunization with self-peptide in the [NOD × NOD.I-A^k]F1 mice resulted either from insufficient I-A^{g7} expression (a gene dose effect), or from heterozygous expression of a (dominant) non-NOD allele in the introgressed H-2^k MHC region. We disproved these possibilities by using [NOD × NOD.I-A^{null}]F1 mice, which express heterozygous levels of I-A^{g7} in the absence of a competing MHC class II gene product, but also (like the [NOD × NOD.I-A^k]F1 mice) possess a significant portion of introgressed non-NOD MHC. We have previously shown that NOD mice mount an autoproliferative response to self-peptide MM110–121 (15). NOD.I-A^k mice showed no response to this peptide (data not shown). [NOD × NOD.I-A^k]F1 mice showed no response to immunization with MM110-121, resembling the parental NOD.I-A^k result (Fig. 2). In contrast, [NOD × NOD.I-A^{null}]F1 mice autoproliferated after immunization with MM110–121 (Fig. 2). This result demonstrated that the quantitative ratio of expression of I-A^{g7} in relation to another MHC class II gene product, rather than the absolute amount of I-A^{g7}, must be critical in forming the bias in the peripheral T cell response in [NOD × NOD.I-A^k]F1 and NOD I-A^k transgenic mice. The autoproliferative response of the [NOD × NOD.I-A^{null}]F1 mice, despite the presence of a heterozygous introgressed non-NOD MHC region, also strongly suggested that the lack of autoproliferative response in the [NOD × NOD.I-A^k]F1 mice was not due to a dominant non-MHC class II gene product in the H-2^k region.

FACS Analysis of Splenic and Thymic APC MHC class II α and β Chain Expression Discriminates NOD I-A^k Transgenic and [NOD × NOD.I-A^k]F1 Mice. To understand how mice with identical NOD background genes (apart from the introgressed Idd1 locus) and the same MHC class II elements could differ so dramatically in their response to immunization with the same self-peptides, we analyzed the expression levels of the I-A^{g7} and I-A^k in the NOD I-A^k transgenic and [NOD × NOD.I-A^k]F1 mice. Peripheral lymph node cells were isolated from the mice, stained with B220 and 39J (I-Aα^k), and analyzed by flow cytometry. PI^+ cells were gated out and $B220^+$ cells were displayed for their MHC class II I-Aα^k levels (Fig. 3 *A*). The NOD I-A^k transgenic and the [NOD × NOD.I-A^k]F1 mice differed in two ways. First, the NOD I-A^k transgenic $B220^+$ cells that were positive for I-Aα^k consistently expressed from two- to fourfold less I-A^k mean channel fluorescence than the [NOD × NOD.I-A^k]F1 mice, under identical staining conditions. Second, the NOD I-A^k transgenic mice reproducibly showed a subset of $B220^+$ cells that lacked appreciable I-Aα^k expression (Fig. 3 *A*, *second row, left column*). Whole peripheral blood FACS® studies using a different antibody, AMS 32.1 (which binds I-Aβ^{g7} but not I-Aβ^k), as well as 39J, showed that the ratio of I-A^{g7} to I-A^k in the NOD I-A^k transgenic mice was reproducibly fourfold greater than the I-A^{g7} to I-A^k ratio in the [NOD × NOD.I-A^k]F1 mice. This was due to the "expected" twofold reduction in the I-A^{g7} (AMS) expression in the [NOD × NOD.I-A^k]F1 mice (compared with the NOD I-A^k transgenic mice; data not shown), combined with the unexpected twofold decrease in I-A^k expression in the NOD I-A^k transgenic (compared with the [NOD × NOD.I-A^k] mice). Whole blood mononuclear cells from NOD I-A^k transgenic mice, in contrast to $B220^+$ cells, did not show a subpopulation of I-A^{g7+}, I-A^{k-} cells.

We next examined I-A^k expression in the NOD I-A^k transgenic versus [NOD × NOD.I-A^k]F1 thymic APCs. Dendritic cells are known to be critical in thymic selection events (21, 22). We isolated thymic dendritic cells from [NOD × NOD.I-A^k]F1 and NOD I-A^k transgenic thymi, using a combination of collagenase digestion, adherence, Ca^{2+} free buffer (23, 24), and, finally, positive selection with CD11c-coated magnetic beads on a Minimacs column. The resultant population of cells was stained with CD11c and MHC class II antibodies and analyzed by flow cytometry. Data presented in Fig. 3 *B* show the MHC class II I-Aα^k (39J) expression of the NOD I-A^k transgenic and

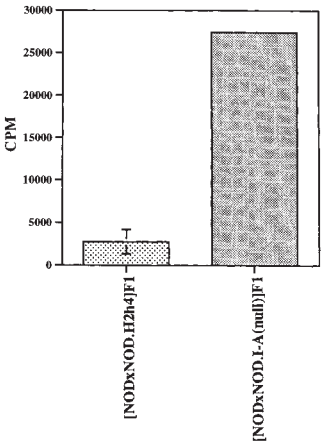
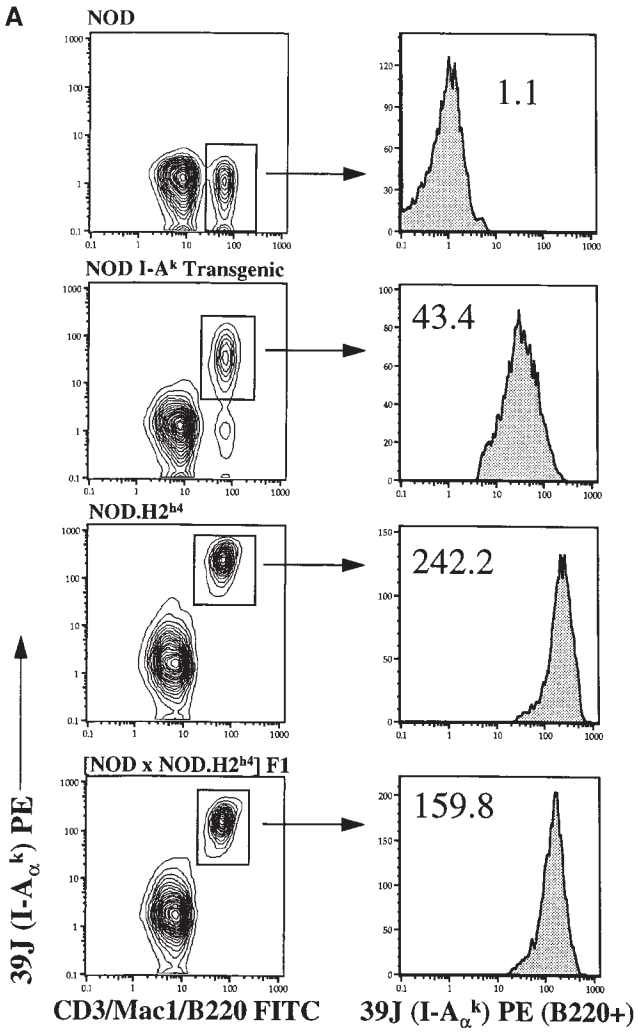


Figure 2. Autoproliferative response in [NOD × NOD.I-A^{null}] F1 versus [NOD × NOD.I-A^k] F1 mice. [NOD × NOD.I-A^k] F1 (n = 5) and [NOD × NOD.I-A^{null}]F1 (n = 2) mice were immunized with self-peptide MM110-121 in CFA; 8 d later draining lymph nodes were cultured in media alone.



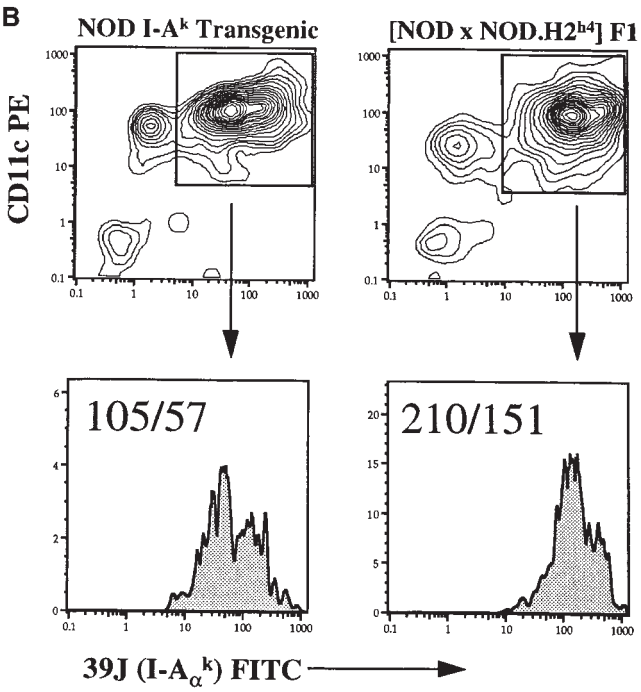


Figure 3. MHC class II expression levels in peripheral and thymic APCs. (*A*) I-Aα^k mean channel fluorescence (numbers in the right column) of B220$^+$ cells from naive peripheral lymph node cells (shown gated in the left column) of NOD, NOD I-A^k transgenic, NOD.I-A^k, and [NOD × NOD.I-A^k]F1 mice. One representative of three experiments is shown. (*B*) I-Aα^k mean (*left*) and median (*right*) channel fluorescence of NOD I-A^k transgenic and [NOD × NOD.I-A^k]F1 thymic CD11c$^+$ cells (shown gated in the *top*). One representative experiment of three is shown.

[NOD × NOD.I-A^k]F1 thymic CD11c$^+$ cells. The thymic CD11c$^+$ NOD I-A^k transgenic I-Aα^k expression reproducibly showed an approximately twofold reduction in the I-Aα^k (39J$^+$) levels compared with that of the [NOD × NOD.I-A^k]F1 mice. Consistent with the peripheral expression, the NOD I-A^k transgenic thymic cells also showed approximately twofold greater expression of I-A^{g7} (AMS mean channel fluorescence) than the [NOD × NOD.I-A^k]F1 cells (data not shown).

I-A^k–restricted T Cell Responses in the NOD I-A^k Transgenic Mouse. A possible explanation of the effect of quantitatively different expression of I-A^k in the NOD I-A^k transgenic and [NOD × NOD.I-A^k]F1 mice was that the level of I-A^k in the NOD I-A^k transgenic mouse was insufficient to mediate some undetermined I-A^k–restricted T cell event. We examined this possibility in two ways. First, NOD I-A^k transgenic CD4$^+$ T cells were shown to be broadly tolerant to I-A^k, despite the decreased I-A^k expression relative to I-A^{g7} (compared with the [NOD × NOD.I-A^k]F1 mouse; Fig. 1 *B*); i.e., there was no MLR reaction of NOD I-A^k transgenic CD4$^+$ cells to NOD.I-A^k APCs. Second, as previously reported for these NOD I-A^k transgenic mice (17), they showed an intact T cell response to immunization with a peptide, HEL 46–61, which binds I-A^k, whereas NOD mice showed no response (data not shown). In these two assays, we did not detect a gross defect in I-A^k peripheral T cell immune function in the NOD I-A^k transgenic mice.

[NOD × NOD.I-A^k]F1 Mice Demonstrate a Poor Response to the I-A^{g7}–restricted Foreign Peptide SWM110–121, Although [NOD × NOD.I-A^k]F1 Peripheral APCs Can Effectively Bind and Present SWM110–121. We addressed the crucial question of whether the demonstrable MHC effect controlling the T cell restriction bias was central (thymic) or peripheral by exploring the response of various mice to immunization with a foreign peptide (SWM 110–121) that differs from the self-peptide MM110–121 by 5 amino acids, is restricted by I-A^{g7}, and does not bind I-A^k. NOD mice responded strongly to immunization with SWM110–121 (Fig. 4 *A*). NOD I-A^k transgenic mice also responded to SWM 110–121, whereas NOD.I-A^k mice showed no response (data not shown). Surprisingly, however, the response to SWM110–121 of [NOD × NOD.I-A^k]F1 mice was much less than the expected semidominant level when compared with the NOD SWM110–121 response (Fig. 4 *A*). To demonstrate that the poor (or absent) response to

SWM110–121 in the [NOD × NOD.I-A^k]F1 mice did not represent a general inability of the [NOD × NOD.I-A^k]F1 I-A^{g7} to bind or present this peptide, we derived a SWM110–121 reactive line from draining lymph nodes of NOD I-A^k transgenic mice immunized with SWM110–121 and assayed the response of T cells from this line to SWM110–121 using irradiated NOD or [NOD × NOD.I-A^k]F1 lymph node cells as an APC source. The [NOD × NOD.I-A^k]F1 APCs efficiently presented SWM110–121 to the NOD I-A^k transgenic T cells, at approximately semidominant efficacy (~50% the response of the homozygous APCs), compared with NOD APCs (Fig. 4 *B*), demonstrating that the [NOD × NOD.I-A^k]F1 peripheral I-A^{g7} had no intrinsic defect in binding or presenting SWM 110–121.

It remained possible that the [NOD × NOD.I-A^k]F1 mice had a defect in I-A^k as well as I-A^{g7} responses, i.e., that there was a global defect in T cell responses in these mice. To test this, we primed NOD.I-A^k and [NOD × NOD.I-A^k]F1 mice with an I-A^k–restricted peptide, HEL 46–61. In contrast to their lack of response to I-A^{g7} restricted peptides (SWM 110–121, MM110–121, MM69–78, and TCR peptide), the [NOD × NOD.I-A^k]F1 mice demonstrated a semidominant response (~50% of the homozygous response) to HEL 46–61 when compared with the parental (homozygous MHC class II–expressing) NOD.I-A^k(Fig. 4 *C*). Thus, the poor (or absent) responses of [NOD × NOD.I-A^k]F1 mice to I-A^{g7}–restricted peptides did not reflect a global defect in $CD4^+$ T cell responses in these mice.

B10.H2^{97} Congenic Mice Lack the Autoproliferative Phenotype. These results demonstrated that the presence of the autoproliferative phenotype was critically dependent upon the quantitative ratio of MHC class II gene expression when the NOD background outside the MHC region was fixed. We next asked whether homozygous I-A^{g7} MHC expression was both necessary and sufficient for the autoproliferative phenotype. We immunized mice expressing homozygous I-A^{g7} on a non-NOD background (B10.H2^{g7} mice) with self-peptide in CFA. The draining lymph node cells of these mice did not autoproliferate (Fig. 5). Thus, homozygous I-A^{g7} MHC expression is necessary, but not sufficient, for the autoproliferative phenotype.

Discussion

Using several lines of "NOD" mice with identical non-MHC NOD background genes, lacking I-E expression and varying in the relative expression of I-A^{g7} and I-A^k, we have studied the quantitative effect of varying MHC class II gene expression on the peripheral T cell response. We have found that the homozygous MHC haplotype determines the response to self-peptides (NOD and NOD.I-A^k responses in Fig. 1 *A*). When two different I-A molecules are coexpressed on the NOD background, the result of immunization with a self-peptide can vary from an autoproliferative response to no response (NOD I-A^k transgenic versus [NOD × NOD.I-A^k]F1; Fig. 1 *B*). The critical determinant of the differences in autoproliferative response in the mixed MHC mice to self-peptide immunization was the relative expression of the two MHC class II molecules (Fig. 3, *A* and *B*). The NOD I-A^k transgenic mice consistently demon-

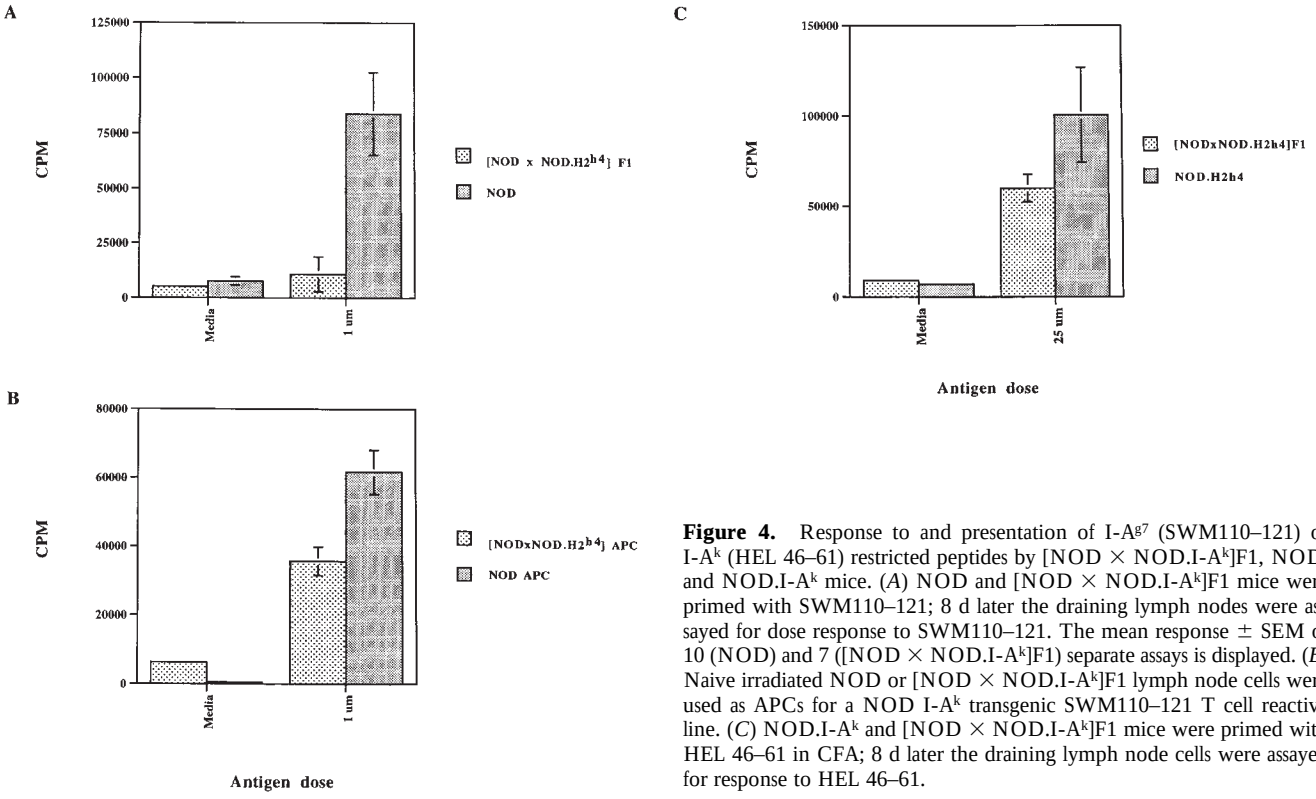


Figure 4. Response to and presentation of I-A^{g7} (SWM110–121) or I-A^k (HEL 46–61) restricted peptides by [NOD × NOD.I-A^k]F1, NOD, and NOD.I-A^k mice. (*A*) NOD and [NOD × NOD.I-A^k]F1 mice were primed with SWM110–121; 8 d later the draining lymph nodes were assayed for dose response to SWM110–121. The mean response ± SEM of 10 (NOD) and 7 ([NOD × NOD.I-A^k]F1) separate assays is displayed. (*B*) Naive irradiated NOD or [NOD × NOD.I-A^k]F1 lymph node cells were used as APCs for a NOD I-A^k transgenic SWM110–121 T cell reactive line. (*C*) NOD.I-A^k and [NOD × NOD.I-A^k]F1 mice were primed with HEL 46–61 in CFA; 8 d later the draining lymph node cells were assayed for response to HEL 46–61.

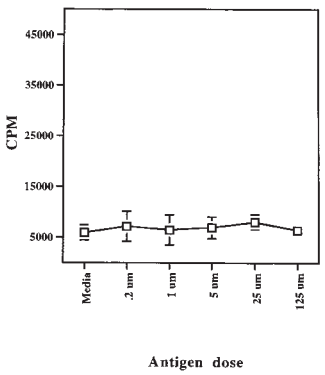


Figure 5. Response of B10.H2^{g7} mice to immunization with self-peptide. B10.H2^{g7} mice were primed with self-peptide TCR in CFA; 8 d later the draining lymph node cells were assayed for response in media ± antigen. The mean of the response of three separate assays ± SEM is shown.

strated two- to fourfold less I-A^k expression in thymic and peripheral APCs than did the [NOD × NOD.I-A^k]F1 mice. The ratio of I-A^{g7} to I-A^k in the NOD I-A^k transgenic mice was at least fourfold greater than that in the [NOD × NOD.I-A^k]F1 mice. The localization of the thymus as the site of the effect of the altered MHC ratio on the peripheral T cell response was demonstrated by studying the responses of [NOD × NOD.I-A^k]F1 mice to peptides that were not presented by (don't bind to) I-A^k, and to which responses were lacking in the NOD I-A^k congenic parent, i.e., SWM110–121 and MM110–121. The poor [NOD × NOD.I-A^k]F1 SWM response (Fig. 4 *A*) could not be due to poor peripheral binding of I-A^{g7} to SWM110–121, since the [NOD × NOD.I-A^k]F1 mice were efficient at binding and presenting SWM110–121 to SWM reactive transgenic T cells (Fig. 4 *B*). The poor [NOD × NOD.I-A^k]F1 SWM response also could not be attributed to insufficient quantity of I-A^{g7} (gene dose effect), since an F1 dose of I-A^{g7} in the absence of a competing "good binding" MHC (in the [NOD × NOD.I-A^{null}]F1 mice) allowed an autoproliferative response to MM110–121 (a response lacking in the [NOD × NOD.I-A^k]F1 mice) (Fig. 2). The autoproliferative response of the [NOD × NOD.I-A^{null}]F1 mice also strongly suggested that the poor [NOD × NOD.I-A^k]F1 response was unlikely to be due to a dominant non-MHC class II gene expressed in the introgressed non-NOD MHC. The overall response to both self- and foreign peptides in the [NOD × NOD.I-A^k]F1 mice suggested a remarkable skewing of T cell restriction toward the parental I-A^k MHC and away from the parental I-A^{g7} MHC. In the absence of a critical ratio of I-A^{g7} to I-A^k expression, autoreactive T cells did not appear at high frequency in the [NOD × NOD.I-A^k]F1 mice, whereas they did appear in the NOD I-A^k transgenic and [NOD × NOD.I-A^{null}]F1 mice. We have not seen an autoproliferative response in any mouse strain other than NOD, suggesting it is a unique response of NOD mice to immunization with self-peptides. Additionally, Kanagawa et al. have recently demonstrated (by limiting dilution analysis) that mice expressing I-A^{g7} have a much higher incidence of autoreactive T cells than do mice expressing other MHC haplotypes (25), strongly suggesting that the results reported here reflect the general T cell repertoire phenotype of I-A^{g7} versus non–I-A^{g7} mice.

The simplest explanation of the data presented here implicates I-A^{g7} in defective thymic selection in a manner directly related to its quantitative expression. In [NOD × NOD.I-A^k]F1 mice, the ratio of MHC expression appears insufficient for effective selection of the I-A^{g7}–restricted peripheral repertoire. The increased ratio of I-A^{g7} to I-A^k in the NOD I-A^k transgenic mice allows selection on I-A^{g7}, but permits escape of T cells with the capacity to recognize and proliferate in response to endogenously processed and presented self-antigens (autoproliferation), as does expression of I-A^{g7} at the homozygous level in NOD mice and at the heterozygous level in absence of a second MHC (in the [NOD × NOD.I-A^{null}]F1 mice), suggesting inefficient negative selection. These results are consistent with previous reports that raised the possibility of defective thymic selection in the NOD mouse. Serreze and Leiter showed that reconstitution of diabetes-resistant [NOD × NON]F1 mice with NOD bone marrow cells eliminated diabetes resistance (26). Moreover, they showed that congenic F1 mice, heterozygous for I-A^{g7} and H-2^{nb1} MHC products, were susceptible to diabetes if reconstituted with NOD bone marrow, whereas reconstitution with an F1 bone marrow resulted in diabetes resistance (26). These results (with an endpoint of diabetes incidence) are compatible with the data presented here on the quantitative effect of MHC class II expression on an autoproliferative T cell response. Deluca et al. published that NOD mice showed the highest ratio of all mouse strains studied of single positive to double positive cells in fetal thymic organ culture, suggestive of less stringent thymic negative selection (27). Forsgren et al. showed that NOD:B6 allophenic chimeras must express >50% NOD MHC phenotype in their lymphoid compartment, as well as express the NOD haplotype in the thymic cortical epithelium, in order to develop insulitis (28). These data suggest that a preponderance of the NOD MHC in the medullary thymic compartment results in defective negative selection (28). Taken as a whole, the literature suggests defective thymic selection in the NOD mouse, and the results presented here suggest that the selection defect may be mediated in a quantitative fashion by relative levels of MHC class II expression.

A mechanism of defective thymic selection by I-A^{g7} is not established here. However, the report that I-A^{g7} is unstable and a "poor peptide binder" (16) suggests a mechanistic explanation when considered in the context of an avidity theory of T cell activation and thymic selection (29–32). Carrasco-Marin et al. showed that I-A^{g7} was unstable in SDS-PAGE analysis, and that this instability correlated with decreased cell surface expression, and found that the peptide binding of I-A^{g7} at acidic pH was too weak to allow kinetic analysis (16). MHC class II instability and poor peptide binding would have a profound effect on thymic selection by decreasing the effective dose of I-A^{g7} MHC–self-peptide (ligand) on the selecting APC surface. In an avidity model of thymic selection (29–32), the net result of universal poor peptide binding by I-A^{g7} (diminished ligand) would be a global increase in TCR affinity for both positive and negative selection in I-A^{g7} homozygous mice. In the [NOD × NOD.I-A^k]F1 mouse, the poor peptide binding of I-A^{g7}, combined with a decreased I-A^{g7} dose,

might dramatically decrease the amount of [I-A^{g7}–self-peptide] available for I-A^{g7}–mediated selection, creating functional "I-A^{g7} clonal ignorance" (33) in the thymus. The presence of the good peptide binder, I-A^k, could enhance the effect by quantitatively superior binding of self-peptides to I-A^k ("determinant stealing", reference 34) in the thymic APCs at the time of positive selection. Notably, although the I-A^{g7} restricted autoreactive response changes dramatically from the [NOD × NOD.I-A^k]F1 to the NOD I-A^k transgenic mouse in response to a change in the effective ratio of I-A^{g7}/I-A^k, the converse is not true; I-A^k–restricted immune peripheral function appears to be grossly intact in the NOD I-A^k transgenic mouse despite a significant decrease in I-A^k expression (Fig. 1 *C*), and I-A^k–restricted responses in the [NOD × NOD.I-A^k]F1 mice are at the expected semidominant level compared with the parental NOD.I-A^k mice (Fig. 4 *C*). Increasing the I-A^{g7}/I-A^k ratio (in the NOD I-A^k transgenic mouse) or I-A^{g7} homozygosity (NOD mice) could overcome the effect of I-A^k when compared with the [NOD × NOD.I-A^k]F1, thereby allowing positive selection of I-A^{g7}–restricted T cells, while the amount of peptide/I-A^{g7} representation in the thymus might still be insufficient (compared with a "good" peptide binding MHC) to mediate effective clonal deletion/negative selection. The result would be the release into the periphery of I-A^{g7}–restricted T cells with higher affinity TCRs than could occur in the thymus of mice with a "good peptide" binding MHC; the increased affinity of the T cells being required to compensate for decreased MHC–peptide density in order to attain the requisite avidity level for selection. (29–32). Once such T cells reach the periphery, the multiple other NOD Idd genes (2) would then interact to allow activation and initiation of a peripheral autoimmune process. That this progression from a high affinity self-reactive (autoproliferative) T cell repertoire to autoimmunity requires more than the "permissive" MHC selection is supported by the lack of an autoproliferative response in non-NOD mice expressing homozygous I-A^{g7} in the absence of sufficient other Idd genes (Fig. 5).

One paradox of any model invoking poor thymic I-A^{g7} peptide binding in defective thymic selection is how T cells escaping deletion could be activated in the periphery, since presumably the same MHC stability defect would be present. A possible explanation involves pH-dependent differential I-A^{g7} stability. Carrasco-Marin et al. reported poor I-A^{g7} peptide binding at pH 5.5 (endosomal), whereas other reports suggested that the binding might be stabilized at pH 7.0 (16, 35). If thymic selection primarily used endogenously processed peptides, the endosomal defect could predominate. In the periphery, however, I-A^{g7} on the cell surface (closer to pH 7.0) at the site of inflammation might stably bind exogenous self-antigens, allowing activation of potentially autoreactive T cells that escaped from thymic selection. However, these theories remain speculative until the normal physiology of thymic selection, and the differences between thymic and peripheral activation of T cells are further elucidated. Nonetheless, our data support defective thymic selection as a mechanism of the unique I-A^{g7}–controlled autoproliferative response.

The model presented here also offers an explanation for the puzzling observation that human and murine diabetes, almost uniquely amongst autoimmune diseases correlated with MHC molecules, require homozygous MHC class II expression for the development of disease. The data on the [NOD × NOD.I-A^k]F1, NOD I-A^k transgenic, and NOD peripheral immune responses suggest that a homozygous dose of MHC class II NOD I-A^{g7}, and possibly human HLA-DQ3.2 (36) is (usually) required for selection of a sufficiently large population of T cells to ultimately mediate autoimmunity. MHC class II heterozygosity would allow a major skewing in the MHC restriction of the expressed peripheral T cell repertoire to a non-self-reactive repertoire due to the role played in thymic selection by the "non-susceptible" MHC class II allele.

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