

High-Density Presentation of an Immunodominant Minimal Peptide on B Cells Is MHC-Linked to Th1-like Immunity

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Ligand-directed differences in the amount of peptide presented on a specific APC subset could influence the functional outcome of any given immune response. We have investigated this issue with a biochemically determined immunodominant peptide that is presented at a higher density on the APC of Th1 responders (I-A^s genotypes) than on the APC of Th2 responders (I-A^b genotypes). MHC-linked high peptide density is expressed on B lymphocytes, predominantly those that bear the B7-2 activation marker/costimulatory ligand. We further investigated the role of I-A^s-specific polymorphism with transfected cells bearing an R→Q change at position-70 of Aβ (found only in the I-A^s allele). Strikingly, I-A^b-restricted Th1 and Th2 clones proliferate at a peptide dose 10- to 100-fold lower than wild-type on transfected fibroblasts bearing this single s-like substitution in Aβ^b. Moreover, the shift in the clone dose response is sensitive to the peptide's C-terminus, as is MHC-linked Th1-like immunity to this peptide *in vivo*. Together, these data suggest that ligand-density can dictate Th1/Th2 selection via a single MHC polymorphism that determines the level of peptide presented to a given TCR on activated B cells. © 1995

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INTRODUCTION

A role for the major histocompatibility complex (MHC) in directing immunity toward cell-mediated versus humoral response pathways is suggested by studies in experimental animals (1–6) and by certain human disease states (7–11). In the mouse immune response to human type-IV collagen, mutually exclusive Th-response patterns are observed (2). Recall CD4 T cell proliferation and IL2 and IFN- γ production are associated with I-A^s genotype mice, whereas I-A^{non-s} strains display recall CD4 T cell help for hapten-spe-

cific B cells and IL4 and IL5 production (2, 12–14). We recently mapped the minimal peptide immunogen capable of eliciting both types of immunity to the $\alpha 2$ (IV) chain, aa 675–686 (14). Truncation of the C-terminus lysine from this 12mer peptide results in decreased recall T cell proliferation and the appearance of helper function from immunized I-A^s genotype mice; and, we demonstrated that this same peptide position controlled the level of peptide presented on splenic APC. We report here that the APC displaying high peptide density are B lymphocytes, predominantly those that are B7-1⁻, B7-2⁺, and show that a single polymorphic position of Aβ^s affects the high versus low dose presentation of this peptide *in vitro*. With regard to the induction of Th1 versus Th2 immunity, these results therefore indicate that a quantitative mechanism (ligand density) is coupled to a qualitative effect (APC type) by the same MHC polymorphism.

MATERIALS AND METHODS

Mice

A.SW/SnJ (I-A^s), A.BY/SnJ (I-A^b) and C57BL/6J (I-A^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.S mice were originally obtained from Dr. C. A. Janeway, Yale University. Mice were age and sex matched for quantitative analyses.

Peptides

Synthetic peptides used in these studies were synthesized and purified by Research Genetics, Inc. (Huntsville, AL) and purity confirmed to >95% homogeneity by mass spectrometry (MassSpec Lab, University of Kansas, Lawrence, KS). The structure of the minimal collagen-IV peptide (12mer) and its otherwise identical C-truncated derivative (11mer) have been previously reported (single letter amino acid code): 12mer, EAIQPGCIGGPK; 11mer, EAIQPGCIGGP (14). Peptides were biotinylated as previously described with a short-chain NHS-biotin (*N*-hydroxysuccinimide-biotin) (Sigma Chemical, St. Louis, MO) (14).

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Generation of CD4 Th Clones

CD4 T cell clones specific for the collagen-IV minimal peptide were prepared from I-A^s and I-A^b congenic mice as previously described (14). The clones reported have been in culture for 1–2 years, have been subcloned at least twice by limiting dilution, and were analyzed for TCR V β expression with a panel of V β -specific monoclonal antibodies (PharMingen, San Diego, CA). Each cloned line is CD4⁺, CD8⁻, and gives a single uniform positive peak fluorescence for only 1 V β of 14 V β analyzed. In three cases, the clone did not react with any of the α V β mAbs tested, indicating a different V β family not tested (14, Shountz and Murray, in preparation).

T Cell Proliferation Assays

The assay has been previously described (14). Briefly, in 96-well plates (Costar, Cambridge, MA), 1×10^5 cells of a given Th clone/well were cultured with synthetic peptides (0.1–100 μ M), and either 5×10^5 APC/well (irradiated syngeneic spleen cells, 2000 R) or 1×10^5 fibroblasts/well (MHC class II transfectants, 3000 R; see below), in Click's medium (Irvine Scientific), containing 2 mM glutamine, 100 U/100 μ g pen/strep, 5×10^{-5} M 2-ME, and 5% FCS (Hyclone, Inc., Logan, UT) (14). Cultures were incubated for 72 hr followed by an overnight pulse with 1 μ Ci/well [*methyl*-³H]thymidine (6.7 Ci/mmol; ICN Biochemicals, Irvine, CA). Triplicate wells were harvested onto glass fiber filters, and incorporated radioactivity was measured by liquid scintillation counting.

MHC Class II Transfectants

The fibroblast lines used were obtained from Dr. R. N. Germain (National Institute of Allergy and Infectious Diseases). The FT6.4 line is transfected with a wild-type A α^b and mutant A β^b combination of cDNA expression vectors (15). The mutant A β^b was generated by oligonucleotide mutagenesis and has a single substitution corresponding to position 70 (changing arginine (R) to glutamine (Q) in the HV3 region of the β 1 α -helix) (15–17). The R→Q change at this position is also a natural polymorphism, which distinguishes the I-A^s allele from all others (16, see Discussion). The wild-type control fibroblast line, FT7.1 (i.e., A α^b /A β^b transfectant), and the untransfected control line, DAP.3, have also been described (15). We confirmed that the transfectants express the B7-1 costimulatory ligand (18) and were found to be B7-2⁻ (data not shown).

Peptide Presentation Density Analysis by Flow Cytometry

Spleen cell suspensions from normal unimmunized mice were prepared from groups of three animals with the ground-glass ends of sterile microscope slides. The

resulting suspension, in HBSS, was fractionated with lymphocyte separation medium (LSM) from Organon Teknika (Rockville, MD). For depletion of specific cell types, the LSM-prep was incubated with either unconjugated anti-B220 (clone RA3-6B2, PharMingen), or anti-CD4 (clone GK1.5), CD8 (clone TIB105), both from ATCC (Rockville, MD). Negative selection of B220⁺ cells used a second antibody (goat anti-rat IgG) conjugated to magnetic microbeads and separation was with the *MiniMACS* following the manufacturer's directions (Miltenyi Biotech, Sunnyvale, CA). T cell depletion was with a 1:15 dilution of low-tox rabbit complement (Accurate, Westbury, NY). For binding analysis, 10^6 viable cells were incubated in round-bottom 96-well plates with increasing concentrations of individual biotinylated peptide antigens at 37°C, 5% CO₂ for 16 hr. All binding reactions were done in sterile 0.5% BSA–PBS, and after binding and each subsequent staining step, the cells were washed three times with 5% FCS–PBS buffer and kept on ice. For staining, avidin–FITC was incubated with the cells on ice for 30 min, followed by biotinylated antiavidin for 1 hr and then again with avidin–FITC. Avidin and antiavidin reagents were purchased from Vector Laboratories (Burlingame, CA). Secondary staining of peptide binding cells was with either anti-B7-1(CD80)–PE-conjugated mAb (clone 1G10), or anti-B7-2(CD86)–PE-conjugated mAb (clone GL1), both purchased from PharMingen. MAb to the B cell marker, CD45R/B220 (clone RA3-6B2) conjugated to Cy-Chrome was purchased from PharMingen and used as the third-tier reagent. After washing, the cells were fixed in 1% paraformaldehyde–PBS and stored at 4°C until analysis. Flow cytometry was performed with a *FACScan* (Becton Dickinson, Mountain View, CA). Data were acquired on 10,000 cells for each sample and analyzed using Lysis II software (Becton Dickinson).

RESULTS

MHC Haplotype Determines the Peptide Dose Response of Distinct TCR-Bearing Th1 and Th2 Clones

Previous results have suggested a ligand density effect upon the selective activation of Th1-like and Th2-like responses (12, 14, 19, 20). We found that the minimal collagen-IV peptide capable of eliciting Th1 immunity in I-A^s mice and Th2 immunity in I-A^b mice was presented at a significantly higher density on splenic APC of the I-A^s genotype (14). In order to determine if single TCR-bearing populations responded to such a ligand density difference, we derived several CD4 Th clones with different TCR from each I-A genotype. As illustrated by the data in Fig. 1, there is a striking conservation in low peptide dose activation in I-A^s-derived clones which is not found among similarly derived

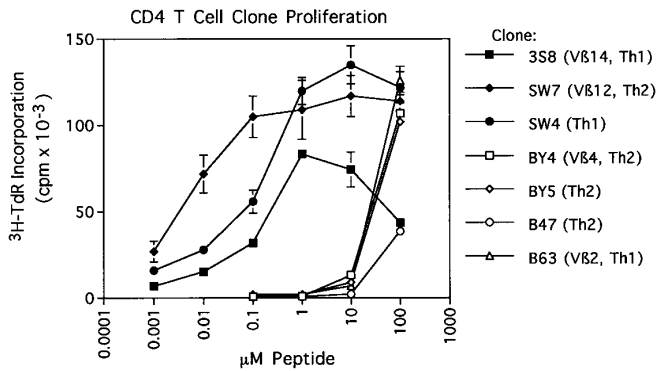


FIG. 1. MHC determines the peptide dose response of distinct TCR-bearing Th1 and Th2 clones on splenic APC. Th1 and Th2 clones were derived from A.SW or A.BY mice immunized with the collagen-IV peptide by standard methods. TCR V β were identified by flow cytometry with a panel of mAb specific for individual V β chains (PharMingen). These various cloned lines were maintained and assayed for recall proliferation to the minimal collagen-IV 12mer simultaneously on MHC-matched splenic APC. Assays were done in triplicate in 96-well flat-bottomed plates in complete Click's medium supplemented with 5% FCS. After 72-hr incubation at 37°C/5% CO₂, individual wells were pulsed with 1 μ Ci [³H]TdR and the radioactivity incorporated over an additional 16-hr incubation was measured by liquid scintillation counting. The I-A^s-derived clones (filled symbols) respond at lower doses of the peptide than the I-A^b-derived clones (open symbols) regardless of their TCR or Th1/Th2 status. These data are representative of five independent experiments.

clones from the congenic I-A^b strain. The finding that several different TCR display this dose discrepancy suggests that TCR affinity is not the factor driving the I-A^s-linked preferential reactivity. Indeed, what would be common to all T cell clones of a given genotype would be the presentation of peptide by that genotype's MHC molecules. Importantly, our clonal analyses have identified that an *in vitro* correlate of the opposing functional outcomes of I-A^s and I-A^b *in vivo* responses against collagen-IV is the quantitative difference in the dose-response curves of Th clones derived from these responses. As previously indicated (14), we have not observed a correlation between the *in vivo* functional response pattern and the types of Th clones generated *in vitro*. However, whatever the Th1, Th2, or Th0 status of the clone, we have always observed proliferation at lower doses of peptide if the clone was derived from an I-A^s genotype mouse.

MHC Haplotype Determines the Density of Peptide on Freshly Isolated Splenic B Cells

To determine if a particular type of APC was responsible for I-A associated differences in the density of peptide presented, we used a combination of multicolor flow cytometry and mAb-negative selection techniques. As is shown in Fig. 2, three-color analysis of the freshly isolated normal cells that bind high density of the peptide reveals that the cells are positive for the pan B

cell marker, B220 (CD45R), and, as expected, peptide density increases on these cells with increasing peptide dose. Note that peptide density on these B cells is significantly different between I-A^s and I-A^b genotypes (compare Fig. 2, left to right). As summarized in Fig. 2, the percentage of double-positive (B220⁺, peptide^{hi}) cells peaks at a dose of 1 μ M peptide and is 28.05% in I-A^s compared to 5.89% in the I-A^b genotype. These are representative data of three separate experiments showing a 5- to 10-fold difference in the frequency of high-density presentation of the peptide on B cells between these two MHC haplotypes.

High-Peptide-Density-Presenting B Cells Express B7-2 Costimulatory Ligand

Since only activated B cells are effective APC for T cell activation (21–23), we analyzed the expression of the activation marker/costimulatory ligand, B7-2 (22, 23), on these high-peptide-density-presenting B cells. As illustrated by the data in Fig. 3 (top left), we find that the I-A^s high-density presenters are predominantly B7-2⁺ (63% in this case). Consistent with their activated phenotype (22, 23), these cells were found to be B7-1 negative (Fig. 4). To confirm these data, we used the B220 marker to deplete the APC pool of B cells by *MiniMACS* technology (Materials and Methods), and these B220⁻ cells were compared for B7-2 and peptide density to the untreated populations (Fig. 3, compare right to left panels). These results confirm that the high peptide presenters are B cells because anti-B220 treatment shows a >97% depletion of these double-positive (B7-2⁺, peptide^{hi}) cells. Note that depletion of CD4- and/or CD8-positive cells did not significantly reduce the percentage of these high-peptide-density-presenting cells when analyzed by identical flow analysis (Fig. 2 and data not shown).

A Single A β^s -Associated Polymorphism Shifts Peptide Dose Responses of I-A^b-Derived Th1 and Th2 Clones

We have noted that the unique ability of the I-A^s genotype to activate Th1-like immunity against collagen-IV is associated with MHC class II presentation of the minimal peptide on B lymphocytes. Thus, one wonders what is unique about the I-A^s molecule that leads to this effect. Sequence comparison between I-A^s and I-A^{non-s} alleles reveals that a position-70 glutamine is found in A β^s , where an arginine is present in all other alleles (15, 16). In order to determine if this specific polymorphism could be responsible for the unique peptide presentation properties of the I-A^s genotype, we analyzed the dose-response curves of nominally I-A^b-restricted Th clones presented with the collagen-IV minimal peptide on mutant (position-70 glutamine) versus wild-type (position-70 arginine) I-A transfected fibroblast lines. As is shown in Fig. 5 (top experiment),

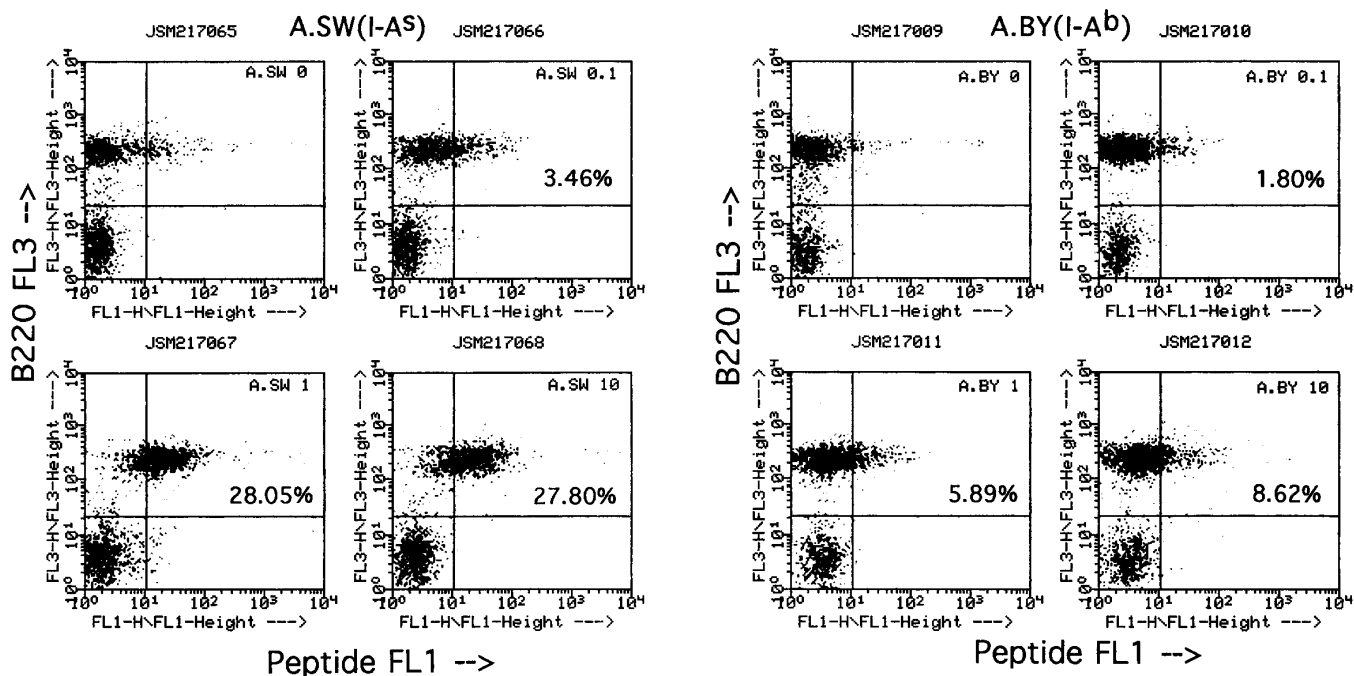


FIG. 2. MHC determines the density of the minimal peptide on B cells. Freshly isolated splenic APC were depleted of CD4/CD8 cells by mAb+ C' treatment, extensively washed, and then incubated for 16 hr with 0.0–10 μ M biotinylated collagen-IV 12mer at 37°C/5% CO₂. Bound peptide was detected with avidin–FITC/biotinylated anti-avidin/avidin–FITC, and these cells were counterstained with a mAb to the pan B cell marker, B220 (CD45R), conjugated to CyChrome. The cells were gated on live lymphocytes by forward/side scatter and data were acquired on 10,000 events for each sample with a *FACScan* and analyzed using *LysisII* software. The peptide titrations for A.SW (left group of four panels) versus A.BY (right group of four panels) cells are shown and the background (i.e., added detection reagents at 0 μ M peptide) percentages of double-positive (B220+, peptide^{hi}) cells has been subtracted from the double-positive percentages at each peptide dose for each MHC haplotype. These are representative data of three separate experiments. As indicated, each axis represents log fluorescence (FL) intensity: B220–CyChrome (FL3), y axis; bio-peptide–avidin–FITC (FL1), x axis.

we found a clear 1–2 log preference for Th proliferation on the s-like mutant fibroblasts. Thus, similar to the situation in Fig. 1, s-like I-A molecules present the peptide more efficiently to single, and different TCR-bearing cells. In this case, the same TCR-bearing population responds at a 1–2 log lower peptide dose on the s-like APC. We wondered if the mutation effects a direct contact between the TCR and the MHC molecule, a contact between the TCR and the peptide, or depends on the density of peptide presented. We used our previous knowledge about presentation and Th responses to a C-truncated derivative of the minimal 12mer to investigate this issue.

The Shift in the I-A^b-Derived Th1 Response to the Position-70 R→Q Mutant Requires the C-Terminal Lysine of the Minimal Collagen-IV 12mer

As is demonstrated in Fig. 5 (bottom experiment), it is clear that the effect of the position-70 s-like substitution must involve the position-12 lysine of the peptide. When the C-terminal lysine is absent from the minimal peptide (right panel, 11mer), there is no augmentation effect of the s-like mutation on Th proliferation (see left panel 12mer control). This result mirrors our ear-

lier work on the presentation densities of the 12mer versus 11mer peptides, where we showed that the 12mer, but not the 11mer can achieve high-density presentation on I-A^s APC (14). Moreover, since the 12mer, but not the 11mer, elicits exclusive Th1-like immunity *in vivo* (14), these data on Ia-transfected fibroblasts confirm that the density of the peptide presented on MHC class II molecules *in vitro* correlates with the *in vivo* Th response pattern. Finally, since the B63 Th1 clone does not recognize the effects of peptide position-12 lysine on wild-type I-A molecules, (compare FT7.1 and splenic APC curves for the 12mer versus 11mer; Fig. 5, left vs right panels), this position is unlikely a TCR contact position (24). However, the s-like molecule may generate an apparent TCR contact involving this position by a conformational effect on the bound peptide (14). Thus, it seems that either ligand density drives the increased reactivity of the B63 clone or the s-like MHC:peptide ligand more effectively contacts the TCR in a manner that requires peptide position 12. With regard to ligand density, this was a peptide-specific effect because staining for total I-A levels showed similar levels on the transfectants and B cells examined (data not shown). While we cannot rule out confor-

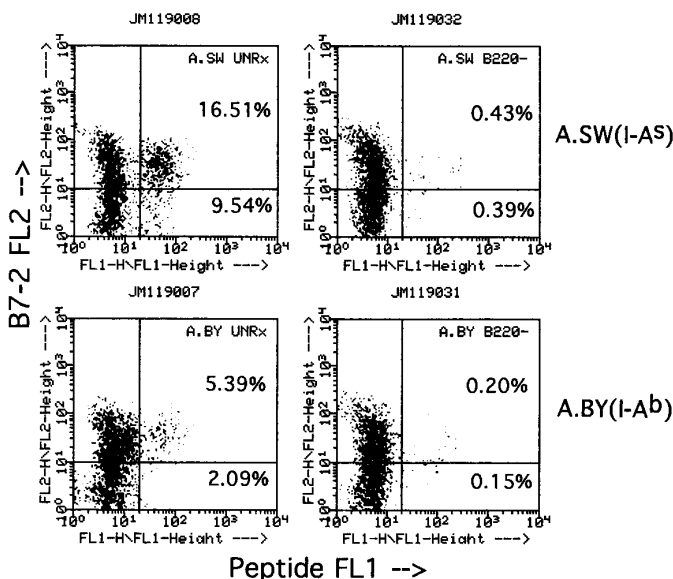


FIG. 3. High-peptide-density-presenting B cells express the B7-2 costimulatory ligand. The B cells presenting high peptide density were analyzed for B7-1 and B7-2 costimulatory ligands. Splenic APC from each MHC congenic strain (A.SW, top versus A.BY, bottom) were incubated with 10 μ M collagen-IV peptide as above and compared for peptide density and costimulatory ligand expression (left panels, top versus bottom). Note that the sum of the peptide-positive percentages approximates the percentage of B220⁺, peptide^{hi} cells shown in Fig. 2. Shown in the right panels (top and bottom), mAb to B220 was used to deplete the population of B cells, and then this depleted population was analyzed as above. As shown, prior B cell depletion results in a >97% decrease in the percentage of B7-2⁺, peptide^{hi} cells. Data are representative of three experiments. B7-2-PE (FL2), y axis; bio-peptide-avidin-FITC (FL1), x axis.

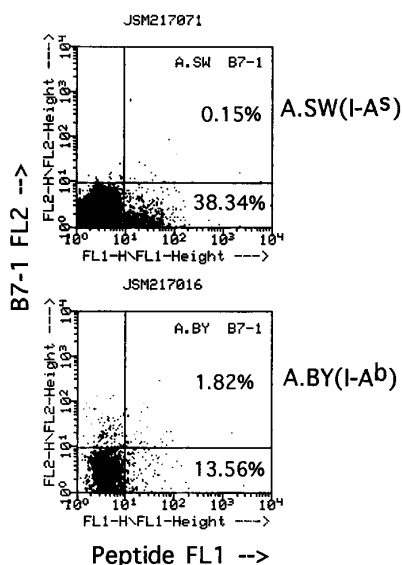


FIG. 4. Lack of B7-1 expression on high-peptide-density-presenting B cells. A.SW and A.BY splenic APC were incubated with the biotinylated collagen-IV peptide as described in the legend to Fig. 3. Three-color analysis was performed as previously described and the peptide binding cells counterstained with B7-1-PE (FL2).

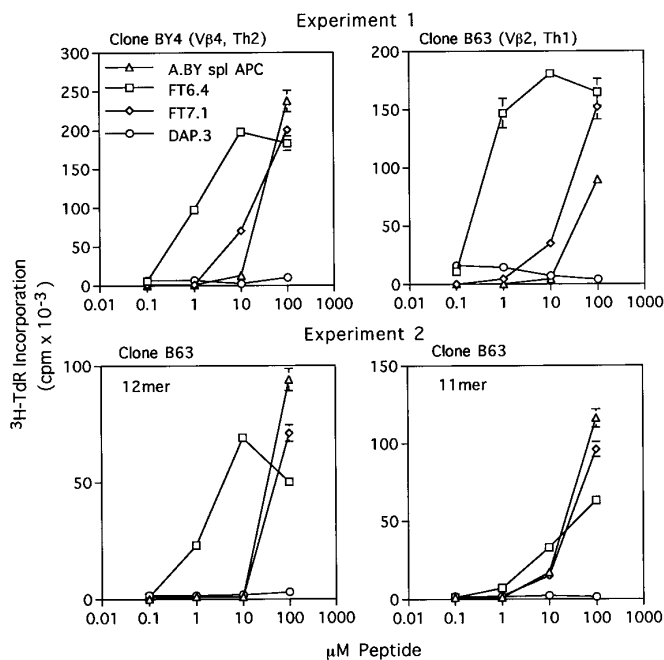


FIG. 5. Transfectants with an A β position-70 “s-like” I-A^b molecule display a peptide-dependent shift in Th clone dose-response curves. The FT6.4 L cell transfectant bears a mutant I-A^b molecule where A β position 70 has been changed from R to Q (a natural polymorphism found only in the I-A^s allele). FT7.1 bears the wild-type I-A^b molecule and DAP.3 is the untransfected control line. These different L cells were compared to A.BY splenic APC for presentation of the collagen-IV peptides to I-A^b-derived Th clones. In the top experiment, the Th2 clone BY4 and the Th1 clone B63 both display a clear 1–2 log preference for the 12mer peptide presented by the s-like mutant MHC. In the bottom experiment, the s-like preference was found to require the 12mer peptide C-terminus, as the truncated 11mer peptide (right panel, 11mer) was similarly presented by both MHC molecules to the same TCR-bearing clone that shows an s-like preference with the 12mer (left panel, 12mer control).

mational effects on the peptide or MHC, on a quantitative basis, the 1–2 log shift in peptide presentation density could account for the 1–2 log shift in proliferation in the absence of altering the intrinsic affinity of the ligand for the TCR. An important question remains, however, with regard to why this *in vitro* effect of peptide-specific ligand density on B cells is associated with selective Th1-like immunity *in vivo*.

DISCUSSION

Focusing high ligand density to activated B lymphocytes might have been an expected aspect of a mechanism which selects for Th1-like immunity. Like dendritic cells, B cell blasts express ligand for costimulation via CD28/CTLA-4 (25). Recent data showed that the frequency of naive T cells capable of responding to peptide presented by activated B cells was high and only slightly lower than dendritic cell presentation (22). This is consistent with an effect of B cell antigen pre-

sentation on recall T cell proliferation, in the absence of an effect on recall helper function (26). Specifically, the high-density presentation observed on I-A^s B cells might serve to shift the frequency of APC usage toward this subset *in vivo*. Further experiments will be required to determine whether the preference in Th1-like priming reflects this shift in APC usage, is a direct result of increasing the avidity of the TCR ligand interaction (27), or requires both MHC-linked effects. Clearly, B cells differ from other APC in the complement of their costimulatory signaling components (22) and could therefore deliver a qualitatively different signal to an uncommitted Th cell at the time of antigen presentation, or when cross-linked by monoclonal antibody to B7-1 versus B7-2 (28). In the collagen-IV system, activated B cells presenting high peptide density express B7-2, but not B7-1, and are associated with exclusive Th1-like immunity. Thus, the distinct endogenous peptide binding motifs of I-A^s and I-A^b molecules (29) could focus the collagen-IV peptide at particularly high levels on I-A^s, but not I-A^b, B cells. The present data indicate that this involves position 70 in the β 1 helix (15–17) and are consistent with previous results which showed that single substitutions in this region effect TCR recognition, but not the expression or overall conformation of the MHC class II molecule (15). Finally, the present results are consistent with high ligand density selective, MHC class II-restricted, suppressor T cells (30, 31). We have shown here that B7-2⁺ B cells from I-A^s mice present the minimal peptide at high density. Previous work demonstrated a lack of CD4 T helper activity for IgG, including IgG2a, in the I-A^s genotype, regardless of high IFN- γ expression in CD4 T cell blasts activated by the cognate collagen-IV antigen (2, 32). Together, these data support a mechanism whereby the profound deficiency of humoral immunity to collagen-IV in I-A^s mice involves Th1-mediated killing, or clonal inactivation, of high peptide density B cell presenters (33, 34). In this regard, the suppression of humoral immunity to some antigens may generally involve peptide determinants like ours which are expressed at high density on activated B cells. Moreover, in cases where selective Th1-like activation is a desirable outcome (e.g., leprosy, leishmaniasis, allergy), targeting high peptide density on B cells would appear to represent a biological route to such an effect.

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