TCR-Independent Pathways Mediate the Effects of Antigen Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy and Paul M. Allen

*J Immunol* 1999; 162:1923-1930; ;
http://www.jimmunol.org/content/162/4/1923
Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy, and Paul M. Allen

We examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR αβ transgenic mouse specific for hemoglobin (Hb)(64-76)/I-Ek. We identified two altered peptide ligands of Hb(64-76) that retain strong agonist activity but, at a given dose, induce cytokine patterns distinct from the Hb(64-76) peptide. The ability of these peptides to produce distinct cytokine patterns at identical doses is not due to an intrinsic qualitative property. Each peptide can induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations and has a unique range of concentrations at which these distinct effects occur. The pattern of cytokines produced from limiting dilution of naïve T cells demonstrated that the potential to develop an individual Th1 or Th2 cell is stochastic, independent of Ag dose. We propose that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of Ag dose involves the modification of soluble factors in bulk cultures that are the driving force that polarize the population to either a Th1 or Th2 phenotype. The Journal of Immunology, 1999, 162: 1923–1930.

TCR-Independent Pathways Mediate the Effects of Antigen Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy, and Paul M. Allen

We examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR αβ transgenic mouse specific for hemoglobin (Hb)(64-76)/I-Ek. We identified two altered peptide ligands of Hb(64-76) that retain strong agonist activity but, at a given dose, induce cytokine patterns distinct from the Hb(64-76) peptide. The ability of these peptides to produce distinct cytokine patterns at identical doses is not due to an intrinsic qualitative property. Each peptide can induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations and has a unique range of concentrations at which these distinct effects occur. The pattern of cytokines produced from limiting dilution of naïve T cells demonstrated that the potential to develop an individual Th1 or Th2 cell is stochastic, independent of Ag dose. We propose that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of Ag dose involves the modification of soluble factors in bulk cultures that are the driving force that polarize the population to either a Th1 or Th2 phenotype. The Journal of Immunology, 1999, 162: 1923–1930.

TCR-Independent Pathways Mediate the Effects of Antigen Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy, and Paul M. Allen

We examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR αβ transgenic mouse specific for hemoglobin (Hb)(64-76)/I-Ek. We identified two altered peptide ligands of Hb(64-76) that retain strong agonist activity but, at a given dose, induce cytokine patterns distinct from the Hb(64-76) peptide. The ability of these peptides to produce distinct cytokine patterns at identical doses is not due to an intrinsic qualitative property. Each peptide can induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations and has a unique range of concentrations at which these distinct effects occur. The pattern of cytokines produced from limiting dilution of naïve T cells demonstrated that the potential to develop an individual Th1 or Th2 cell is stochastic, independent of Ag dose. We propose that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of Ag dose involves the modification of soluble factors in bulk cultures that are the driving force that polarize the population to either a Th1 or Th2 phenotype. The Journal of Immunology, 1999, 162: 1923–1930.

TCR-Independent Pathways Mediate the Effects of Antigen Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy, and Paul M. Allen

We examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR αβ transgenic mouse specific for hemoglobin (Hb)(64-76)/I-Ek. We identified two altered peptide ligands of Hb(64-76) that retain strong agonist activity but, at a given dose, induce cytokine patterns distinct from the Hb(64-76) peptide. The ability of these peptides to produce distinct cytokine patterns at identical doses is not due to an intrinsic qualitative property. Each peptide can induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations and has a unique range of concentrations at which these distinct effects occur. The pattern of cytokines produced from limiting dilution of naïve T cells demonstrated that the potential to develop an individual Th1 or Th2 cell is stochastic, independent of Ag dose. We propose that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of Ag dose involves the modification of soluble factors in bulk cultures that are the driving force that polarize the population to either a Th1 or Th2 phenotype. The Journal of Immunology, 1999, 162: 1923–1930.

TCR-Independent Pathways Mediate the Effects of Antigen Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy, and Paul M. Allen

We examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR αβ transgenic mouse specific for hemoglobin (Hb)(64-76)/I-Ek. We identified two altered peptide ligands of Hb(64-76) that retain strong agonist activity but, at a given dose, induce cytokine patterns distinct from the Hb(64-76) peptide. The ability of these peptides to produce distinct cytokine patterns at identical doses is not due to an intrinsic qualitative property. Each peptide can induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations and has a unique range of concentrations at which these distinct effects occur. The pattern of cytokines produced from limiting dilution of naïve T cells demonstrated that the potential to develop an individual Th1 or Th2 cell is stochastic, independent of Ag dose. We propose that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of Ag dose involves the modification of soluble factors in bulk cultures that are the driving force that polarize the population to either a Th1 or Th2 phenotype. The Journal of Immunology, 1999, 162: 1923–1930.

TCR-Independent Pathways Mediate the Effects of Antigen Dose and Altered Peptide Ligands on Th Cell Polarization

Arash Grakoui, David L. Donermeyer, Osami Kanagawa, Kenneth M. Murphy, and Paul M. Allen

We examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR αβ transgenic mouse specific for hemoglobin (Hb)(64-76)/I-Ek. We identified two altered peptide ligands of Hb(64-76) that retain strong agonist activity but, at a given dose, induce cytokine patterns distinct from the Hb(64-76) peptide. The ability of these peptides to produce distinct cytokine patterns at identical doses is not due to an intrinsic qualitative property. Each peptide can induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations and has a unique range of concentrations at which these distinct effects occur. The pattern of cytokines produced from limiting dilution of naïve T cells demonstrated that the potential to develop an individual Th1 or Th2 cell is stochastic, independent of Ag dose. We propose that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of Ag dose involves the modification of soluble factors in bulk cultures that are the driving force that polarize the population to either a Th1 or Th2 phenotype. The Journal of Immunology, 1999, 162: 1923–1930.
then further backcrossed to B6.AKR containing the H-2k allele to introduce the appropriate restriction element. They were then crossed to RAG1-/- mice to abrogate expression of endogenously rearranged receptors (16). The average thymus size from 2.102 TCR Tg RAG-/- mice is 1 × 10⁶ cells, and the average number of splenocytes is 3 × 10⁹ cells.

Antigens

The variants of Hb(64-76) agonist peptide are referred to by a one-letter code representing the substituted amino acid followed by its position. For example, K69 refers to the Hb(64-76) peptide that has lysine substituted for threonine at position 69. The names and sequences of the peptides used in this study are as follows: Hb(64-76), GKKVITAFNEGLK; D73, GKKVITAFNDGLK; K69, GKKVIKAQNEGLK; and S70, GKKVITSNFEGLK. All peptides were synthesized on an Applied Biosystems (Foster City, CA; model 432A) or a Rainin (Woburn, MA) Symphony Multiplex peptide synthesizer. The peptides were purified by reverse-phase HPLC and their amino acid composition was confirmed by mass spectrometry (Washington University Mass Spectrometry Facility, St. Louis, MO) and amino acid analysis (Beckman (Fullerton, CA) model 6300) (17, 18).

Cell lines

The generation and characterization of the Th cell clone, 2.102, and the hybridoma cell line, G2, have previously been described (5, 11, 19). T cell lines from the 2.102 TCR Tg RAG-/- mice were propagated as described (20) using 2.5 × 10⁵ Tg splenocytes and 5 × 10⁵ irradiated (2000 rad) non-Tg B6.AKR splenocytes as a source of APC in a 24-well culture plate. During the primary culture, splenocytes from the 2.102 TCR Tg RAG-/- mice were stimulated with various doses of Hb(64-76) or APLs. On days 3–4, cells were expanded threefold into fresh medium in 24-well plates. On day 7, the T cells were harvested, washed, counted, and restimulated at 2.5 × 10⁵/well by 5 × 10⁵ B6.AKR splenocytes presenting 1–10 μM Hb(64-76) peptide. Supernatants were collected at 48 h, and the cytokine profile was assayed by ELISA as described below. Tissue culture medium was RPMI 1640 containing 10% FCS (HyClone, Logan, UT), 2 mM Glutamine, 0.5 mM Hβ(64-76) in the presence of APCs lacking CD4 and CD8. Subsequently, T cells were stained with phycoerythrin-conjugated anti-CD4 or fluorescein-conjugated anti-CD8 (PharMingen, San Diego, CA) for FACS analysis.

FIGURE 1. 2.102 TCR is efficiently selected on the RAG−/−H-2k haplotype. Thymocytes (A through C) or splenocytes (D through F) from the 2.102 TCR Tg RAG−/− mice on k/k (A and D), k/b (B and E), or b/b (C and F) haplotypes were stained with CD4 and CD8. As illustrated, the selection of thymocytes and peripheral Tg T cells progressively increased in the H-2k haplotype compared with H-2k/b heterozygote littermates (compare A with B and D with E). In the nonselecting H-2k background, there was no evidence of selection of single positive thymocytes (C) nor of any peripheral T cells (F). Based on other markers such as CD62L and CD25, the vast majority of the peripheral TCR Tg T cells in the selecting backgrounds have a naive phenotype (data not shown). The values in the panels represent the percentages of the cell population. This figure is representative of more than five experiments.

Flow cytometric analysis

Cells (1 × 10⁶) were incubated at 4°C for 1 h with 100 ng of either phycoerythrin-conjugated anti-mouse CD4 or fluorescein-conjugated anti-mouse CD8 (PharMingen) in a total volume of 200 μl of staining buffer (PBS containing 2% BSA). Cells were then washed, resuspended in 400 μl of staining buffer and 1% paraformaldehyde, and analyzed by flow cytometry on a Becton Dickinson FACScaliber (Becton Dickinson, Franklin, MA).

Results

2.102 TCR is efficiently selected on the RAG−/−H-2k haplotype

The 2.102 TCR Tg mouse was generated as described in Materials and Methods. Fig. 1 compares the CD4/CD8 profiles of thymus cells and splenocytes between different haplotypes of 2.102 TCR Tg RAG−/− mice. T cells expressing the 2.102 TCR were efficiently positively selected on the H-2k haplotype. We had thus generated a TCR Tg mouse, through the use of which we could examine Th cell differentiation in the Hb(64-76) system.

The response of the 2.102 TCR Tg RAG−/− splenocytes to Hb(64-76) and D73 recapitulates the results with 2.102 T cell clone

We wanted to confirm the cytokine profile produced by D73 and Hb(64-76) in a more physiologic setting. Previously, it was shown...
that stimulation of the 2.102 T cell clone with D73 peptide induced IL-4 production in the absence of proliferation (5). Therefore, we extended our observations to the Tg mice expressing the same TCR. Naive 2.102 TCR Tg RAG-2/2 cells were stimulated with 1, 0.1, or 0.01 μM of Hb(64-76); 100 μM of D73 peptide; or 1 μM ionomycin plus 50 ng/ml PMA. After 1 wk, equivalent numbers of all the T cells were restimulated with 1 μM of Hb(64-76). As shown in Fig. 2, D73 ligand induced a strong Th2 response in the 2.102 TCR Tg RAG-2/2 splenocytes, even though the proliferation seen by this peptide during primary stimulation was diminished more than 10,000-fold in comparison with Hb(64-76). Our result with Hb(64-76) stimulation is consistent with the data reported in other systems (7, 8), in that, with a low dose of Ag, the phenotype of the T cells is polarized toward a Th2 phenotype, and with higher Ag doses, toward a Th1 response.

The response of the 2.102 TCR Tg RAG-/- splenocytes to other APLs can be compared with the Hb(64-76) response

To examine a broader spectrum of proliferative responses of the 2.102 TCR Tg RAG-/- splenocytes, we used a panel of APLs that have substitutions at critical residues interacting with the TCR (11, 24). To assess the phenotypic development of these Tg T cells after the primary stimulation, we concentrated on ligands that had either agonist or weak agonist properties. The previously described D73 ligand is a weak agonist ligand and requires a very high Ag dose (100 μM) to stimulate the T cells. We needed to find other altered ligands for which we could vary the concentration during the primary culture. Since we were unable to vary the concentration of D73, we used K69 and S70 for subsequent experiments. As shown in Fig. 3A, stimulation of the Tg T cells with Hb(64-76) and K69 resulted in very similar proliferative responses, with K69 slightly more stimulatory at lower concentrations of peptide. Stimulation with the S70 ligand resulted in a proliferative response that was shifted about fivefold from the response to Hb(64-76). All these ligands have their maximal proliferative response at 1 μM of each peptide.

To rule out the possibility that the substitution of amino acids within the wild-type peptide did not alter the affinity of that peptide for the MHC, an assessment of this interaction was made by measuring direct binding capacity to the purified I-Ek molecule (25).
and assayed for the presence of IL-4. Supernatants were harvested 48 h after secondary stimulation for each of these three ligands was maximal at 1 μM concentration of peptide, yet IL-4 production at this concentration produced when naive 2.10² TCR Tg RAG knockout splenocytes were restimulated with 100 μM of Hb(64-76) peptide after 1 wk of propagation with Hb(64-76) or variants of it. Supernatants were harvested 48 h after secondary stimulation and assayed for the presence of IL-4 (top) or IFN-γ (bottom) by ELISA. This figure is representative of more than three experiments.

The results indicated that Hb(64-76), K69, S70, and D73 bind to the MHC with identical affinities (data not shown). We therefore eliminated the variability in Ag presentation of these ligands to the T cell.

Variants of Hb(64-76) induce different cytokine patterns

Previous reports have indicated that an APL can induce a cytokine pattern that differs from that induced by the wild-type agonist ligand (9, 10, 26). The presence or absence of IL-2 absolutely did not result in any difference in phenotype development of our bulk cultures (data not shown). Shown in this report, primary stimulation was done in the absence of any exogenous cytokines, and production of IL-4 or IFN-γ was assessed after secondary stimulation with Hb(64-76). Fig. 4 depicts values for IL-4 or IFN-γ produced when naive 2.10² TCR Tg RAG knockout splenocytes were stimulated with 1 μM wild-type or APL for 1 wk and subsequently restimulated with Hb(64-76). As Fig. 3 shows, proliferative responses for each of these three ligands was maximal at 1 μM concentration of peptide, yet IL-4 production at this concentration was very different (Fig. 4).

The primary Ag dose affects cytokine production

Next, we wanted to examine whether the differential cytokine production we observed with APLs was due to an intrinsic ability of the peptide to transduce a qualitatively different signal to the T cell. We altered the concentrations of the peptides used during the primary stimulation as shown in Fig. 5 and assessed IL-4 production. The results indicated that although there was a lack of IL-4 produced by K69 at a 1 μM concentration of peptide (Figs. 4 and 5), decreasing the concentration of K69 during the primary culture increased IL-4 production (Fig. 5). While it is at 1 μM of each peptide that the proliferative responses seen for each of the three ligands is maximal, it is clear that the concentration for optimal cytokine production for each peptide differs. Lower concentrations are optimal for K69-stimulated IL-4 production than for S70 or Hb(64-76). This argues against the idea that each ligand has an absolute intrinsic ability to drive Th1 or Th2 differentiation, since for each given peptide, a dose can be found at which it will stimulate specific cytokine production. This study is consistent with those reported by others, in which it has been shown that low doses of peptide Ags will induce a Th2 response, while higher doses will direct the differentiation toward a Th1 phenotype (7–10). However, these results also show that for each given ligand, there is a range of concentrations at which cells will be driven toward Th1 or Th2, but the concentrations at which these occur are not necessarily identical for each given ligand.

Increasing Ag dose results in more IFN-γ-producing T cells

We then wanted to investigate whether the differences in cytokine production seen in the bulk populations result from stimulating different percentages of cells, each of which produces a constant amount of cytokine, or from stimulating a constant percentage of cells that produces a fluctuating amount of cytokine. To address this, we used the intracellular staining of T cells for IL-4 or IFN-γ after restimulation. The controls consisted of either no stimulation or stimulation in the presence of exogenous IL-4 or IL-12 for Th2 and Th1 development, respectively. As shown in Fig. 6A, if cells were not stimulated, no IL-4 or IFN-γ-producing T cells were detected. However, upon primary Ag stimulation in the presence of exogenous IL-4, we detected about 13% IL-4-producing T cells and essentially no IFN-γ-producing cells. In the presence of exogenous IL-12, about 23% of cells produced IFN-γ, and no cells made IL-4. The amounts of cytokines produced under these conditions based on the ELISA assay correlate very well with the percentages of different IL-4- or IFN-γ-producing T cells. In a Th2 environment, a large amount of IL-4 is made, and no IFN-γ is made. In a Th1 response, we detected a large amount of IFN-γ and no IL-4 (Fig. 6A). In the case of different doses of Hb(64-76) stimulation in the absence of any exogenous cytokines, we observed that at low peptide stimulation there was a higher percentage of cells producing IL-4 (Fig. 6B). At extremely high concentrations, we detected more IFN-γ-producing T cells. Interestingly,
Subsequently, the cells were stained intracellularly for IL-4 and IFN-γ presence of exogenous cytokines to drive toward a Th2 or Th1 phenotype. This figure is representative of two experiments.

To further examine the role of IFN-γ peptide stimulation or were stimulated with 1 μM of D73 or 1 μM of S70 or K69 in the presence of APCs lacking any CD4+ or CD8+ T cells. Cells were subsequently fixed and stained for intracellular IL-4 or IFN-γ as described in Materials and Methods. This figure is representative of two experiments.

FIGURE 6. Intracellular staining for IL-4 and IFN-γ on 2.102 TCR Tg RAG−/− cells. In A, naive 2.102 T cells were cultured in the absence of any peptide stimulation or were stimulated with 1 μM of Hb(64-76) in the presence of exogenous cytokines to drive toward a Th2 or Th1 phenotype. Subsequently, the cells were stained intracellularly for IL-4 and IFN-γ production. The supernatants from these cultures were also assayed for cytokines by ELISA and corresponded with a complete Th2 or Th1 polarization. In B, T cells were stimulated with varying doses of Hb(64-76) or at 100 μM of D73 or 1 μM of S70 or K69 in the presence of APCs lacking any CD4+ or CD8+ T cells. Cells were subsequently fixed and stained for intracellular IL-4 or IFN-γ as described in Materials and Methods. This figure is representative of two experiments.

FIGURE 7. Increasing the IFN-γ-producing cells during the primary stimulation promotes Th1 development. Naïve 2.102 TCR Tg RAG−/− splenocytes (5 × 105) were cultured in the presence of APCs and different doses of Hb(64-76) peptide. In A, cultures contained either 50 μg/ml of anti-IFN-γ (H22) or no added Abs. In the presence of H22, T cells do not undergo Th1 development. In B, cultures contained either 1000 U/ml of exogenous IFN-γ across all doses of primary peptide stimulation or no exogenously added cytokines. Addition of IFN-γ induces Th1 development even at low doses of peptide stimulation and dramatically decreases the IL-4 production by T cells. This figure is representative of two experiments.

also consistent with the proliferation data shown in Fig. 3A, significantly increased numbers of cells were invariably recovered from primary cultures stimulated with higher Ag doses (data not shown). However, there is a dose of Hb(64-76) ligand at which both IL-4- and IFN-γ-producing T cells can be detected at similar percentages. With D73 at 100 μM and S70 at 1 μM stimulations, there was a skewing toward the IL-4-producing cells. However, K69 stimulation at 1 μM favored increased percentages of IFN-γ cells. Therefore, rather than having a constant number of cells increasing or decreasing cytokine production, different stimulation conditions resulted in changing percentages of cells producing each cytokine.

Blocking IFNγ produces exclusively Th2 cells, while addition of IFNγ augments differentiation of Th1 cells

To further examine the role of IFN-γ during Th cell differentiation, we added either 50 μg/ml of anti-IFN-γ (H22) or 1000 U/ml of exogenous IFN-γ to the primary stimulation with different doses of Hb(64-76) (Fig. 7). Although after restimulation there was not a remarkable difference observed in IL-4 production across the Ag doses in the presence of H22, the level of IFN-γ production was dramatically decreased, even at a high Ag dose. In the presence of H22, T cells exclusively produced Th2 cytokines. In the presence of exogenously added IFN-γ, we no longer attained a Th2 phenotype, even at the low doses of peptide stimulation, as observed previously. There was a dramatic decrease in the production of IL-4, with an increase in IFN-γ production by the T cells across all of the Ag doses. These data support the idea that increasing the amount of IFN-γ in the primary culture leads to Th1 phenotype development. In combination with the results shown in Fig. 6B, this suggests that Th1 development is dependent upon a critical number of cells producing IFN-γ activated in the primary culture.

The ability of T cells to produce IL-4 or IFN-γ at the single-cell level is stochastic and independent of Ag dose

We wanted to understand whether the differential cytokine pattern seen due to the effect of Ag dose on bulk cultures was reflected at the level of single T cells. To address this issue, we performed limiting-dilution assays and stimulated the individual T cells with different concentrations of Hb(64-76) and a fixed number of APCs. As shown in Fig. 8A, in contrast to the bulk culture stimulation, independent of Ag dose, we identified both IL-4- and IFN-γ-producing T cells. Even under low- or high-dose conditions, under which we only observe IL-4 or IFN-γ produced at a population level, we saw that at the single-T cell level, this event was stochastic. Further, the potential of a given T cell to develop into a Th1- or Th2-type cell was independent of the stimulatory dose.

Presumably, under limiting-dilution conditions, we have eliminated the cross-talk that normally exists between the T cells and APCs. Therefore, as a control we wanted to confirm that under the conditions of the limiting-dilution assay, exogenous factors would still influence Th cell differentiation. As shown in Fig. 8B, stimulation of single T cells in the presence of exogenous cytokines...
such as IL-4 or IL-12 directed the phenotype development toward Th2 or Th1, respectively. Taken together, our results show that Th1 and Th2 development is determined at the population level, at which the establishment of cross-talk between T cells and APCs exists.

There is a threshold for generation of a Th1 population
In the bulk culture stimulation, there is polarization of cytokine production depending on the Ag dose or the APL used. One possible explanation is that APLs do not deliver an intrinsically distinct signal through the TCR, but rather act by having different thresholds for cellular activation in bulk culture. We therefore propose a simple model in which there is a threshold, correlating with the percentage of IFN-γ-producing cells, that determines the overall Th1/Th2 phenotype of the population. Below this threshold of stimulation with any ligand, a Th2 response predominates, whereas above this threshold a Th1 response is favored. There exists a zone in which both Th2 and Th1 cells coexist. To distinguish the possibilities, we measured the percentage of the T cells producing IFN-γ as a function of Ag dose (Fig. 9). At low concentrations of Hb(64–76), the percentage of the IFN-γ cells was very low, whereas at higher peptide concentrations the number of IFN-γ-producing T cells increased rapidly. The results of the studies using APLs correlate well with this scenario. For example, at 100 μM stimulation with D73 peptide, the majority of the cells activated were IL-4-producing, leading to the generation of a Th2 phenotype at the population level. The same is true for S70 stimulation at 1 μM of Ag. However, as with the Hb(64–76) peptide, we observed that increasing the dose of the S70 ligand would activate enough IFN-γ-producing cells to shift the bulk culture toward a Th1 response. At 1 μM of K69, the threshold of IFN-γ-producing cells had already been reached. Therefore, by lowering its concentration, we could then induce a Th2 phenotype.

Discussion
We were interested in dissecting the role of Ag dose and APLs in Th cell differentiation. We have used Hb(64–76) as our model Ag system; the crystal structure of this epitope with I-Ek has been elucidated (24). We also took advantage of TCR Tg mice that were crossed to RAG1-deficient mice to eliminate any possibility of expression of endogenously rearranged TCRs. Our results indicated that at low doses of Ag, IL-4-producing cells dominate and thereby drive the T cells toward a Th2 phenotype. In contrast, at high Ag doses, IFN-γ-producing cells dominate and a Th1 response is generated. APLs may require lower or higher Ag doses to give the same phenotype as the wild-type ligand but show the same basic dose dependence. For the very weak agonist ligands, such as D73 in this study, the Ag dose necessary to reach the threshold for induction of a Th1 phenotype may not be attainable. We propose that it is not an absolute intrinsic ability of individual ligands that causes Th1 or Th2 development, but rather that the activation of a critical number of cells at high doses results in the production of enough IFN-γ in the milieu to effect polarization to Th1.

We believe that our results unify much of the data that has been put forth by other colleagues. Several studies have shown that, in the case of soluble Ags, a Th2 response results at low doses of priming Ag, and a Th1 response occurs with higher Ag concentrations (27). In the OVA system, Hosken and colleagues showed that low Ag doses (<0.05 μM) generated Th2 effector cells, intermediate doses induced Th1 cells, and high doses (100 μM) produced Th2 cells (7). It is difficult to directly compare our system with the OVA model and other systems because of the different genetic backgrounds of the mice. However, one similarity in these systems is that the endogenous IL-4 that is produced is absolutely

FIGURE 8. Development of a Th1 or Th2 phenotype at the single-cell level is independent of primary Ag dose. Naive 2.102 TCR Tg RAG−/− splenocytes (50–100 cells/well) were cultured in the presence of APCs (5 × 104 cells) and different doses of Hb(64-76) peptide in 96-well U-bottom plates for 10 days. The cultures contained 50 U/ml rIL-2. Subsequently, the wells that were positive plates for 10 days. The cultures contained 50 U/ml rIL-2. Subsequently, the wells that were positive

FIGURE 9. Threshold for development of Th1 cells. Upon stimulation with low doses of Hb(64-76), 100 μM D73, or 1 μM of S70, lower percentages of IFN-γ producing T cells were detected. However, at higher doses of Hb(64-76) or with 1 μM of K69 stimulation, the percentages of IFN-γ-producing cells increased rapidly, thereby switching from the Th2 to a Th1 phenotype. Open squares (□) depict Hb(64–76) stimulation.
critical for Th2 development at low doses of Ag stimulation, since neutralizing Abs to IL-4 also abrogate this phenotype in our system (data not shown) (7, 27). By our limiting-dilution experiments, we find that with Th1/Th2 the phenotype choice is independent of Ag dose (Fig. 8), suggesting that the dose effects are not due to direct alteration in the quality of signals delivered to the T cells. Rather, this result is instead consistent with the hypothesis that the dose effect is mediated by altering the cytokine milieu. Kamogawa et al. have shown that upon T cell activation, a naive cell expresses the IL-4 gene and gives rise to both IL-4 and IFN-γ-producing cells (28). In our limiting-dilution assay, we were able to detect both IL-4 producers and IFN-γ producers across a wide spectrum of Ag dose. Together it seems that stochastic differentiation of a T cell to produce IL-4 or IFN-γ is more favorable than a preselected phenotype of Th1 vs Th2 cells. The critical issue to address is the discrepancy between cytokines produced at the single-cell level and those made by a bulk culture. Our explanation is that, at low doses of Ag, fewer total cells are activated in the primary stimulation, and thus the concentrations of T cell-derived cytokines available in this case would be quite low. At this stage, perhaps the level of IL-4 that is produced in the culture is dominant over IFN-γ or the IL-12 from macrophages. The autocrine effects of IL-4 could then induce a selective advantage for the survival or development of Th2 cells. At higher doses, enough T cells are activated that the level of IFN-γ is sufficient to override Th1 inhibition by IL-4 and induce Th1 development. We observed that by neutralizing IFN-γ, cells lost the capacity to undergo Th1 development. However, neutralizing IFN-γ did not augment the ability of cells to produce IL-4. Addition of exogenous IFN-γ promoted acquisition of the Th1 phenotype even at low doses at which the Th2 phenotype had previously been observed. Thus, IFN-γ promotes the ability of T cells to become Th1 cells and disfavors the development of Th2 cells at every Ag dose. These data support the model that increasing the number of IFN-γ-producing T cells in the primary culture promotes Th1 differentiation. We also envision that there may exist a dose at which IL-4- and IFN-γ-producing cells can exist simultaneously (Fig. 6B).

An effect of APLs in influencing Th phenotype was previously reported for a T cell clone (26, 29). Studies by Bottomly and colleagues have shown that, in the human collagen IV and moth cytochrome c systems, APLs can selectively skew the population toward either the Th1 or Th2 phenotype (9, 10, 30). In these studies, the differential effects were attributed to the induction of qualitatively distinctive biochemical signals by APLs. If development of Th1 vs Th2 were dependent upon the qualitative nature of the signal delivered, then one would predict that the Th polarization effect would be unaltered across the entire range of doses of a given ligand. However, by altering the dose of any given APL, we are able to change the phenotype of the response. However, for extremely weak agonists such as D73, a sufficient dose for Th1 development is not attainable. Although qualitative effects of APLs cannot be completely excluded, previous observations with variant ligands may be explained by quantitative effects of Ag dose on the alteration of the cytokine milieu.

Our intracellular cytokine staining results correlate with previous reports (31, 32), in which Bucy and colleagues observed a higher frequency of cytokine mRNA-expressing T cells with increasing Ag dose. However, a difference in the intensity of the staining, using their system of digoxigenin-labeled riboprobes, with the individual cells across the range of antigenic doses was not seen. Accordingly, our limiting-dilution assay shows that far fewer cells are activated at low doses, but those that do respond give similar amounts of IL-4 or IFN-γ and proliferation as individual cells from high Ag dose stimulation. Thus, our conclusions support a simple model in which Th1 development in vitro requires a larger total number of cells making IFN-γ, thus allowing the culture to reach a critical threshold for cytokine production. Once the amount of cytokine in the medium passes this threshold level, the bulk population is then polarized toward a Th1 response.

Acknowledgments
We thank Holly Hanson and Devraj Basu for their invaluable help in critically reading this manuscript. We also thank Mark M. Davis for the purified I-Eβ and Shirley J. Petzold for the help with the peptide binding determinations. We sincerely thank Darren Kreamalmeyer, Kathy Frederick, and Donna Thompson for their invaluable help with screening and maintaining the TCR Tg mouse colony. We thank Steve Horvath for his expert technical assistance in synthesis and purification of the peptides and Jerri Smith for assistance in preparation of the manuscript.

References