Modulation of T Cell Development by an Endogenous Altered Peptide Ligand

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Summary

T cells potentially encounter numerous endogenous peptides during selection in the thymus and in the periphery. We examined the impact of an endogenous peptide on in vivo T cell development, using a TCR transgenic mouse model based on a hemoglobin-specific T cell clone. In these mice, the transgenic β chains paired with endogenous α chains. This led to a serendipitous primary reactivity to Ser69 peptide, an altered peptide ligand of the Hbd (64-76) epitope of the parent clone. Two Ser69-reactive T cell populations were identified. A smaller population of the Ser69-reactive T cells responded both to Ser69 and Hba (64-76). A majority reacted only to Ser69, and not to Hba (64-76); in fact, Hba (64-76) was a specific TCR antagonist for these Ser69-only-reactive T cells. Thus, in this unique experimental system, Ser69 became an agonist, and Hba (64-76) was an antagonist. Endogenous presentation of the antagonist ligand in the thymus selectively eliminated the high-avidity cells, while sparing low-avidity cells in the Ser69-reactive T cell repertoire. These results highlight how specificity guides developing T cells through a network of ligands and indicate that the endogenous peptide pool has a profound effect on T cell development and repertoire.

Materials and Methods

TCR-transgenic Mice. The parent clone of the G2Tg mice was the Hb(64-76)/I-Ek-specific Th2 clone, 2.102, derived from a CE/J (Hbb) mouse (7). Mini-genes of the 2.102 TCR-α and -β chains were constructed by insertion of V-Jα and V-D-Jβ exon cassettes into α and β transgene shuttle vectors, respectively (kindly provided by M. Davis (Stanford University, Palo Alto, CA) (8), then coinjected into (B6 × SJL)F2 zygotes at DNX Corp. (Princeton, NJ). Founder transgenic mice (G2-Tg) were obtained and backcrossed twice to CE/J mice to introduce the I-Ek restriction element and maintain homozygosity of the Hbb allele. PCR amplification of the α transgene from tail digests identified transgenic progeny (9). Mice used in the experiments described in this report were homozygous H-2k by FACS or a PCR-based screen.

Initial examination of thymocytes and splenocytes revealed no relative increase in CD4+ T cells in the transgenic progeny and a weak response to Hb(64-76). The TCR-β chain transgene, a Vβ1-Db1-Jβ2.4 rearrangement, was functional and resulted in suppression of endogenous β chain rearrangements in >95% of T cells, as determined by flow cytometry. In regard to the α chain (Vα2-Jα41) transgene, no increase in Vα2+ T cells in G2-Tg mice was detected by an anti-Vα2 antibody (10). Another α transcript (Vα4-Jα45) subsequently was isolated from the parent 2.102 clone that bore strong homology to α chains isolated from other Hbb-specific T cells, suggesting that the Vα2 chain used to make the transgene was not involved in Hb recognition.

Peptides. The peptides used in these studies were synthesized, purified, and analyzed as previously described (7). The peptide sequences (in single-letter amino acid code) are as follows: Hb(64-76), GKKVITAFNEGLK; Ser69, GKKVISAFNEGLK; Gln72, GKKVITAFQEGKL; and Cβ, NGKEVHSGVSTKQAYKE.

T Cell Hybridomas. Unprimed Tg LN cells were activated in vitro for 4 d with Ser69 peptide (15 μM) and fused with the BW5147ot/ ot- thymoma (11). Clonal hybridomas were tested for Ag specificity on CH27 APC as described (7).

T Cell Proliferation and Antagonism Assays. Primary LN and spleen cells were cultured for 4 d, pulsed with 1 μCi (100 μL) [3H]-thymidine, and harvested, and [3H]-thymidine incorporation was determined.

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cell proliferation assays were performed as described (7, 11). TCR antagonist assays of T cell hybridomas were performed as described (4). The antagonist assay for primary T cells was done similarly except that mitomycin C-treated, DCEK.H7 I-E–bearing L cells (5) pulsed with 20 μM Ser69 were the stimulatory APC, and nylon wool-purified T cells (2 × 10^8) from HBb^α1 TCR-transgenic mice were used.

**Sequence Determination of TCR.** For sequence determination of endogenous TCR-α chains, total RNA was isolated from 1–4 × 10^6 T hybridoma cells, and 2 μg RNA was reverse transcribed by use of random hexamers according to manufacturer's instructions for the RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT). PCR was done in a thermocycler TempBionic (Thermolyne Corp., Dubuque, IA) on 1/20 of the reverse-transcription reaction using 15 pmol Vα primer and 20 pmol Cα primer in a 50-μl reaction volume. Primers used were Vα1, Vα4, Vα5/7, Vα6/12, Vα8, Vα11, Vα15 (12); Cαα, Vα2, Vα3, Vα9, Vα10, Vα34S-281, VαBMA, VαBMB, VαA10, Vα13.1, VαBWB, VαST (13, 14); and Vα21 primer 5′-CAGCGCTGTCATCAACTGCA-3′ (15).

Reactions were heated to 94°C for 3 min, followed by four cycles of 97°C for 60 s, 52°C for 30 s, 72°C for 60 s, and 30 cycles of 94°C for 45 s, 52°C for 30 s, and 72°C for 60 s. PCR reactions (7 μl) were electrophoresed on a 3% agarose gel (NuSieve; FMC Corp., Rockland, ME) and visualized with ethidium bromide. Each Vα PCR reaction yielding a band of the expected size was purified by filtration through a microconcentrator (Amicon, Beverly, MA), and then directly sequenced with a nested Cα sequencing primer (generously provided by K. Murphy, Washington University, St. Louis, MO) using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a sequencer (model 373A; Applied Biosystems). TCR-α chain rearrangements were determined to be in frame or out of frame of the V-Jα junctional region sequences. All TCR-α V-J junctions differed at the nucleotide level. Vα and Jα gene segments were assigned according to previous nomenclature (16, 17). The Vα2 transgene was transcribed in all of the hybridomas. Expression of the Vβ1 transgene, which had been transcribed with the α transgene, was verified for all hybridomas by activation by staphylococcal enterotoxin A (Toxin Technology, Sarasota, FL) and/or RNA PCR of the mature transcript of the β transgene.

**Results and Discussion.** To explore the effect of APL on unmanipulated primary T cells, we generated a TCR–transgenic mouse based on the HBb(64-76)/I-E–specific Th2 clone 2.102 (7). Upon characterization, these G2-Tg mice were phenotypically TCR-β chain only–transgenic mice, because the TCR-α chain was not the one used by the 2.102 T cells for HBb(64-76) recognition. Thus, the T cells in the G2-Tg mice expressed a transgenic β chain paired with endogenous α chains. Further characterization of the G2-Tg mice revealed that the transgenic β chain alone, in combination with endogenous α chains, was sufficient to confer antigen reactivity on unprimed T cells. Transgenic spleen cells responded weakly but specifically to either HBb protein or HBb(64-76) peptide (Fig. 1). A variety of APL of HBb(64-76) were also assayed, given the possibility that TCR formed by the transgenic β chain and endogenous α chains would recognize the variants better than HBb(64-76) itself. Interestingly, G2-Tg spleen cells proliferated strongly to Ser69 (Fig. 1). The vigorous proliferative response to Ser69 indicated that the β chain alone had shaped the repertoire to contain a high frequency of Ser69-reactive T cells. Similar antigen responsiveness of unprimed cells has been reported in another TCR-β chain only–transgenic model (18).

To characterize the molecular basis for the Ser69 response, we generated a panel of Ser69-reactive T cell hybridomas from unprimed G2-Tg T cells stimulated in vitro with Ser69 and examined both specificity and TCR-α chain usage. These hybridomas separated into two major groups based on their specificity for Ser69 and HBb(64-76). One group (6/10) could be activated by Ser69 only, not HBb(64-76), and predominantly expressed Vα6-Jα48 TCR-α rearrangements (Fig. 2A). The other group (4/10) consisted of T cells that were activated by both Ser69 and HBb(64-76), and half of these expressed Vα4-Jα33 TCR-α rearrangements (Fig. 2B).

Although the larger group of Ser69-reactive hybridomas was not activated by HBb(64-76), it was still possible that these T cells engaged HBb(64-76), but in a nonfucific manner. Nonstimulatory peptides may act as specific antagonists of the response to the immunogenic ligand (4, 19). Thus, HBb(64-76) was examined as a TCR antagonist of a representative Ser69-reactive hybridoma. For BT, a Vα6-Jα48–bearing hybridoma, HBb(64-76) was not an agonist (Fig. 2A), but did antagonize Ser69-mediated activation in a specific and dose-dependent manner (Fig. 2C), while a control peptide did not. This finding demonstrated that although the BT TCR was activated only by Ser69, it still could specifically interact with the HBb(64-76) peptide. Thus, in the G2-Tg mouse, the roles of these two peptides had been reversed from the parent 2.102 clone (4), with Ser69 now being an
agonist and Hb\(^\beta(64-76)\) an antagonist. This reversal provided us with an in vivo model to address the important question of how a TCR antagonist peptide expressed in the thymus would affect T cell development.

The important role of peptide-specific interactions in T cell development was demonstrated in recent reports (20–22). Peptides that could positively select in vitro in a TCR-\(\alpha/\beta\)-transgenic system were TCR antagonists of the parent T cell clone (20). Peptides that could negatively select would stimulate the parent clone (20, 21). Extrapolating from these findings, we contend that each thymocyte must face a continuum of different potential ligands, and the effects these various ligands have on developing T cells need to be clarified.

To study the effect of an endogenous antagonist on T cell development, we crossed the Ser69-reactive G2-Tg mice with CBA/J mice, which express the Hbb\(^d\) allele. We had shown previously that in Hbb\(^d\) mice, endogenous Hbb\(^d/\beta\)-I-E\(^k\) complexes are constitutively expressed on thymic cortical epithelial cells and medullary APC populations (23), establishing that these complexes would be available to interact with developing CD4\(^+\) T cells.

The presence of the Hbb\(^d\) ligand altered the repertoire. In Hbb\(^d\) mice, vigorous T cell responses to Ser69 were observed, as had been found in Hbb\(^d/\beta\) background mice. In marked contrast, however, cells from Hbb\(^d/\beta\) mice failed to respond to Hb\(^d(64-76)\) (Fig. 3 A). These results suggested that the endogenous Hbb\(^d\) ligand deleted at least all Hb\(^d(64-76)\)-reactive cells in the thymus, whereas Ser69 only-reactive cells were allowed to develop. To gauge whether the endogenous Hbb\(^d\) ligand depleted the transgenic T cell repertoire of any and all cells that interacted with it, Hb\(^d(64-76)\) was assayed as a TCR antagonist peptide. As shown in Fig. 3 B, the primary Ser69 response by Hbb\(^d/\beta\) G2-Tg cells was strongly and specifically inhibited by Hb\(^d(64-76)\) peptide, indicating that the Ser69-reactive T cells were capable of recognizing the ligand as an antagonist, and that a single peptide ligand can inhibit an oligoclonal T cell response. Furthermore, our results clearly show that TCR antagonism of unprimed peripheral T cells can occur.

Comparison of the Ser69 dose response of transgenic T cells from Hbb\(^d/\beta\) with that of Hbb\(^d/\beta\) mice revealed that a higher dose of Ser69 was required to elicit a proliferative response from Hbb\(^d/\beta\) cells (Fig. 3 C). This shift in dose responses were remarkably consistent, in that the data shown in Fig. 3 C were obtained from four individual mice for each group, each assayed on different days. Thus, the observed differences represented a significant shift in the primary response of bulk T cells to Ser69. The elimination of Ser69/Hb\(^d(64-76)\) dual-reactive cells only accounts for a minor proportion of this decreased response of the Hbb\(^d/\beta\) G2-Tg cells, because in Hbb\(^d/\beta\) mice these cells are a minority of Ser69 responders (Fig. 2), and the weak Hb\(^d(64-76)\) response (Fig. 1) suggests a similarly limited contribution by dual-reactive cells to the overall Ser69 response.

The large shift in the Ser69 response in Hbb\(^d/\beta\) G2-Tg mice could be due in part to the endogenous Hbb\(^d\) in the thymus further restricting the Ser69-reactive repertoire to only low-avidity T cells. To test this possibility, the CD4 dependency of the cells was evaluated. Antibody to CD4 more readily inhibited the Ser69 response of G2-Tg spleen cells from Hbb\(^d/\beta\) mice than that from Hbb\(^d/\beta\) mice (Fig. 3 D), indicating that Hbb\(^d/\beta\) cells were more dependent on CD4 coreceptor for activation. This result suggested that the Ser69-reactive cells that emerged in Hbb\(^d/\beta\) G2-Tg mice did recognize Ser69 with lower avidity. The Ser69-specific cells that matured were otherwise similar to cells from Hbb\(^d/\beta\) mice (Fig. 2 A), in terms of TCR-\(\alpha\) usage and precursor frequency (data not shown) and in their recognition of Hb\(^d(64-76)\) as a
TCR antagonist (Figs. 2 B and 3 B). Overall, these results indicated that the endogenous APL selectively removed the high-avidity component of the T cell response to Ser69, but allowed the remaining low-avidity Ser69-reactive cells to develop normally.

Could the peripheral T cell response to Ser69 be affected by antagonism by the endogenous Hbb^d ligand on APC? We have yet to observe any antagonism by endogenous Hbb^d complexes of Ser69 responses of purified T cells, either from Hbb^{d/s} or Hbb^{d/s} G2-Tg mice (data not shown). The number of complexes may be a factor, since TCK antagonism is observable under carefully titrated conditions of suboptimal agonist concentrations and antagonist excess (19). Also, differences between the endogenous ligand and synthetic Hbb^d(64-76) peptide in either length, register, or conformation within the MHC binding groove could translate into differences in antagonist ability. Jameson et al. (24) have recently shown that mature T cells limit their response to the positive selecting ligand by decreasing the expression of CD8 molecules. Thus, in the Hbb^{d/s} mice, perhaps surface levels of CD4 are decreased as part of the regulation in preventing the endogenous Hbb^d (64-76) from antagonizing the Ser69 response. The CD4 level may not need drastic downregulation to see an effect, given that expression of TCR and other coreceptors also may determine whether antagonism occurs. We would argue that, in the periphery, most endogenous peptides can act as antagonists only under unique circumstances such as increased expression of the endogenous peptide or the upregulation of important molecules for T cell activation.

The multiple interactions between Hbb^d(64-76) and different TCR in this system demonstrate that the capacity to finely discriminate between very similar ligands can govern the fate of a T cell. We propose the following model, depicted in Fig. 4, to explain our data. In the thymus, for each TCK, a corresponding set of functionally similar “lookalike” ligands defines a narrow avidity window for positive and negative selection (25, 26). Of note, the Hbb^{d/s} thymus differs from the Hbb^{d/s} thymus because the added endogenous Hbb^d determinant changes the avidity window for selection. In the Hbb^{d/s} thymus, the two specificity types of Ser69-reactive cells (Fig. 2) are positively selected on their respective sets of ligands and proceed to mature. TCR affinity, TCR and coreceptor levels, and determinant density all contribute to
Thymic Selection

Hbb\textsuperscript{s/s} thymus

- Ser69
- Potential ligands
- Ser69/Hbb\textsuperscript{(64-76)} dual-reactive

Hbb\textsuperscript{d/s} thymus

- Ser69
- High Avidity
- Hb
- Potential ligands
- Ser69/Hbb\textsuperscript{(64-76)} dual-reactive

Periphery

- Ser69
- Hbb\textsuperscript{(64-76)} Antagonizes

Figure 4. A proposed model for the effect of the endogenous APL, Hbb\textsuperscript{(64-76)}, on the development of the Ser69 response. In G2-Tg mice, two Ser69-reactive populations of T cells occur: Ser69 only and Ser69/Hbb\textsuperscript{(64-76)} dual-reactive. In the thymus of Hbb\textsuperscript{s/s} mice, these two populations encounter many potential ligands from the endogenous peptide: MHC pool, some of which are positively selecting (P). The positively selected T cells mature and exit the thymus. In the periphery, the Ser69 only-reactive T cells can be antagonized by the Hbb\textsuperscript{(64-76)} synthetic peptide. In the thymus of Hbb\textsuperscript{d/s} transgenic mice, both T cell populations encounter numerous ligands, including the endogenous Hbb\textsuperscript{d} ligand (Hb). The dual-reactive cells and high-avidity Ser69-reactive cells engage the Hbb\textsuperscript{d} ligand sufficiently to induce deletion. Low-avidity Ser69-reactive T cells, which are more antigen and CD4 dependent, develop and exit into the periphery. Like their Hbb\textsuperscript{s/s} background counterparts, these low-avidity Ser69-reactive peripheral T cells from Hbb\textsuperscript{d/s} mice can be antagonized by the exogenous Hbb\textsuperscript{d}\textsuperscript{(64-76)} synthetic peptide.

avidity and influence thymic selection (21, 27–30). Thus, both the Ser69/Hbb\textsuperscript{(64-76)} dual-reactive cells and higher avidity Ser69 only–reactive cells engage the endogenous Hbb\textsuperscript{(64-76)} ligand enough that they are negatively selected. Other Ser69-reactive cells do not interact strongly with endogenous Hbb\textsuperscript{d} and are still positively selected because their avidity window has not changed.

After exiting into the periphery, mature T cells appear to have higher thresholds of activation than thymocytes (31). Thus, ligands that are weak agonists for peripheral T cells are very effective at inducing thymic deletion (31). Similarly, since Hb\textsuperscript{(64-76)} interacted with peripheral T cells as a TCR antagonist in Hbb\textsuperscript{s/s} G2-Tg mice (Fig. 3 B), these cells probably were more sensitive to the endogenous Hbb\textsuperscript{d} ligand during thymic development. However, this interaction was too weak (in affinity or avidity) or nonfickacious to induce thymocyte deletion (26). In fetal thymic organ culture studies, it has been demonstrated that lower avidity TCR interactions allow positive selection, and higher avidity ones result in negative selection (20, 21). In the G2-Tg mice, the endogenous Hbb\textsuperscript{d} ligand may act as a positively selecting peptide look-alike for Ser69-specific cells. Other endogenous peptide look-alikes must serve to positively select as well, since Ser69-specific cells emerge in Hbb\textsuperscript{d/s}-transgenic mice, which lack Hbb\textsuperscript{d} ligand. Such endogenous ligands would probably function as TCR antagonists; however, we contend that TCR antagonism is not directly equated with the ability to positively select. TCR antagonist activity is principally an in vitro property displayed by a class of ligands. In the continuum of interactions between a TCR and different APL, positive selection and antagonist peptides represent separate but overlapping portions of the spectrum (1).

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