Brief Definitive Report

Minor H Antigen HA-1–specific Regulator and Effector CD8+ T Cells, and HA-1 Microchimerism, in Allograft Tolerance

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Abstract

The role of the hematopoietic lineage-restricted minor histocompatibility (H) antigen HA-1 in renal allograft tolerance was explored. We obtained peripheral blood samples from three recipients of histocompatibility leukocyte antigen (HLA)–matched, HA-1–mismatched renal transplants, one of which had discontinued immunosuppression >30 yr ago while sustaining normal kidney function. Peripheral blood mononuclear cells (PBMCs) were injected into the footpad of severe combined immunodeficiency mice to measure human delayed type hypersensitivity (DTH) responses. All three patients manifested regulated DTH responses to HA-1 peptide. By differential tetramer staining intensities, we observed two distinct minor H antigen HA-1–specific CD8+ T cell subsets. The one that stained dimly had the characteristics of a T regulatory (T R) cell and produced interleukin (IL)-10 and/or transforming growth factor (TGF)-β. These HA-1–specific T R cells coexisted with bright tetramer-binding CD8+ T effector (T E) cells. The CD8+ T E cells mediated HA-1–specific DTH and produced interferon-γ. Suppression of these T E functions by T R cells was TGF-β, IL-10, and cytotoxic T lymphocyte–associated antigen 4 dependent. In addition, HA-1 microchimerism was detected in two recipients, primarily in the dendritic cell fraction of the PBMCs. This is the first demonstration of coexisting CD8+ memory T R and T E cells, both specific for the same HA-1 antigen, in the context of renal allograft tolerance.

Key words: regulatory T cells • immunoregulation • peripheral tolerance • kidney transplantation

Introduction

The basis of immunologic tolerance remains largely a mystery >50 yr after its discovery by Owen (1). Inhibition of potentially pathogenic self-reactive T cells by CD4+ T regulatory (T R) cells has been proposed as one of the major mechanisms for the establishment of peripheral tolerance to autoantigens (2, 3) and for the persistence of certain forms of chronic infection (4). Transplantation tolerance studies in rodents have suggested that certain donor-derived alloantigens, which stimulate T cell–dependent graft rejection, could also trigger T R cell responses that promote allograft acceptance (5, 6). However, the T cell receptor specificity of T R cells and what drives their development remain largely unknown.

The hematopoietic-specific minor histocompatibility (H) antigen HA-1 is a nine–amino acid peptide encoded by a diallelic gene on human chromosome 19 (7, 8). The immunogenic HA-1 T cell epitope differs from its allelic counterpart by one amino acid at position 3 (i.e., VLHDLLEA→VLRDDLLEA; reference 7). Although nonameric peptides of both the HA-1H and the HA-1R alleles bind to HLA-A2, the HA-1R allele fails to be expressed at the cell surface in the context of HLA-A2 (7). The HA-1H peptide is presented at the cell surface and induces strong HLA-A2– restricted CTLs (9, 10). The HA-1 difference between HLA-A2+ bone marrow donor and recipient, when the recipient is H/R or H/H, and the donor is R/R, can lead...
to the development of acute GVHD (11). HA-1–specific mismatch GVHD occurs early after bone marrow transplant when residual recipient APCs can still provide the target antigen, triggering bystander destruction of skin and other epithelial tissues (12).

HLA-identical siblings and HLA-matched cadaver donors are the ideal renal transplant donors and indeed have the best outcomes long-term. Yet, in the case of a minor H antigen such as HA-1, the efflux of large numbers of “passenger leukocytes” (13) early after transplantation might induce activation of donor-specific cytotoxic and proinflammatory T effector (Teff) cells that could trigger bystander destruction of the kidney epithelium. In contrast, long-term persistence of donor-derived microchimerism (14) might lead to chronic suppression of host Treg cells (15, 16). Here, we demonstrate the coexistence of CD8+ Treg and Teff cells, both specific for the same hematopoietic-specific minor H antigen, HA-1, together with dendritic cell microchimerism in the context of solid organ transplant tolerance.

Materials and Methods

Animals. CB-17 SCID mice were purchased from Harlan Sprague Dawley, Inc. or were bred locally. All animals were housed and treated in accordance with National Institutes of Health guidelines.

Reagents, Antibodies, and Antigens. All antibodies used in flow cytometry were purchased from BD Biosciences. Neutralizing anti–human CD152 (CTLA-4) mAb was purchased from Antibody Solutions. IL-10– and TGFβ-neutralizing antibodies were purchased from R&D Systems. HA-1H, HA-1R, CMVpp65 (NLVPMVATV), and HY (FIDSYICQV) peptides were all synthesized and purified (purity >94%) at the University of Wisconsin-Madison Biotechnology facility. HA-1A2 tetramers were prepared as described previously (11). All other reagents were purchased from Sigma-Aldrich.

Patients. Patient I (tolerant; 32 yr off immunosuppressive drugs; HLA A 2, 3; B7, 12/44; DR1, 4; and HA-1 R/R) received a kidney transplant from her HLA-identical sister (HA-1H/H) in 1967. Patient II (HLA: A1, 2; B8, 27; DR4, 17; and HA-1R/B) received a kidney transplant from her HLA-identical HA-1–mismatched brother (HA-1H/R) in 1996. Patient III (HLA: A2, 24; B35, 57; DR4, 11; and HA-1R/B) received an HA-1–identical, HA-1–mismatched kidney transplant from a sister in 1988, lost the graft 10 yr later because of chronic allograft nephropathy, and received a second HA-1–mismatched transplant from a 5-HLA antigen-matched sister (HLA: A2, 24; B44, 57; DR4, 11; and HA-1H/R) in 2000. Control patient IV (HLA: A2, 24, B13, 35; DR7, 8; and HA-1: R/R) received a kidney transplant from her HLA- and HA-1R/R–identical brother in 1997. Patients II and IV are taking azathioprine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil. All patients currently have prine and cyclosporine, and patient III is taking prednisone, tacrolimus, and mycophenolate mofetil.

Results and Discussion

We tested DTH responses to the HA-1H antigen in the footpads of SCID mice. PBMCs were obtained from 4 HLA-A2+, HA-1–R/R recipients of primary renal transplants from HLA-identical sibling donors, including one (patient III), who was tested 3 yr after a second transplant from a sibling matched for HLA-A, -DR, and for 1 (out of 2) HLA-B alleles. Patient I stopped taking azathioprine and prednisone 5 yr after transplant (19) and remained off immunosuppressive drugs with excellent graft function for >30 yr (17). The other three allograft recipients are still taking immunosuppressive drugs. As shown in Fig. 1 A, all PBMCs tested had weak swelling responses to the HA-1H peptide, either in the presence of PBS (unpublished data) or a control IgG. In the three patients who received a HA-1H–mismatched renal transplant, DTH responses to HA-1H peptide were observed when neutralizing antibodies to either IL-10 (patient II), TGFβ (III), or both (I) were connected. The control recipient, patient IV, who received a HA-1A2 kidney transplant, remained unresponsive to HA-1H after cytokine neutralization (Fig. 1 A). The HA-1H–responsive TR cells also demonstrated “linked suppression” of DTH response to a recall antigen (Fig. 1 B). Al-
though PBMCs of patients I–III had strong DTH responses to TT or EBV antigen alone, these responses were markedly suppressed in the presence of coinjected HA-1H peptide. In contrast, patient IV’s PBMCs retained a strong response to recall antigens in the presence of HA-1H (Fig. 1 B).

The DTH response of tolerant patient I to HA-1H was donor specific and dose dependent, with no anti-HY (third party) or anti-HA-1R (self) peptide responses seen in the presence of neutralizing anti-TGFβ antibodies (Fig. 1 C).

**Regulator and Effector HA-1-specific T Cells Can Be Distinguished by HLA Tetramer Staining Intensity.** The recovery of a strong DTH response to HA-1H by cytokine neutralization suggested that PBMCs obtained 36 yr after renal transplant contained not one, but two distinct functional subtypes of HA-1H-specific T cells as follows: one mediating DTH, the other producing TGFβ and/or IL-10, thereby suppressing DTH. Two types of minor H antigen-specific T cells were distinguishable by HA-1A2 tetramer (11) staining intensity. Fig. 2 A shows that the majority of the small CD8+ T lymphocytes that stained positively (1.22 ± 0.46%; n = 5) showed low tetramer staining (HA-1A2-low; mean fluorescent index = 58 ± 25). A small portion (0.056 ± 0.027%; n = 5) of the CD8+ T cells showed bright staining (HA-1A2-high; mean fluorescent index = 720 ± 160). Next, sorted HA-1A2-high and HA-1A2-low CD8+ T cells were tested for DTH response to HA-1H peptide. As shown in Fig. 2 B, control mouse footpad injections of PBMCs depleted of tetramer-binding CD8+ cells (TDPs) plus HA-1H peptide caused a weak swelling. In the presence of TDP and peptide, 10⁵ HA-1A2-high CD8+ T cells caused a strong DTH response, whereas 3 x 10⁴ HA-1A2-low CD8+ T cells failed to mediate a detectable swelling response. When combined with the HA-1A2-low cells, the DTH response of HA-1A2-high cells was suppressed. TDP still contained CMV-reactive CD8+ T cells, as indicated by a strong DTH response to CMVpp65 (Fig. 2 B). Addition of tetramer-sorted, HA-1A2-low CD8+ T cells had no effect on the DTH response to CMVpp65 (Fig. 2 B), which confirmed that, in the absence of cognate ligand for the CD8+ T cell, there is no linked suppression of a third party antiviral CD8 T cell response.

The suppression by HA-1A2-low CD8+ T cells of the HA-1-specific DTH response of HA-1A2-high CD8+ T cells was reversed by addition of TGFβ neutralizing antibody, but not by a control IgG. The magnitude of the recovered DTH response was dependent on the dose of added HA-1A2-high T cells (Fig. 2 C). Furthermore, the HA-1H peptide, but not CMVpp65, induced approximately twofold increased expression of intracellular TGFβ1 over the background level in sorted HA-1A2-low CD8+ T cells during overnight culture with autologous APCs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20031012/DC1). To see if the CD8+ T cells could respond to endogenous HA-1 antigen at levels normally expressed by donor leukocytes, we used a linked suppression system similar to that shown in Fig. 1 B, except that B-LCLs were used as a source of both EBV and endogenous minor H antigen. We found similarly strong DTH responses to autologous B-LCLs and to EBV antigen; however, a markedly reduced response to donor B-LCLs was observed (Fig. 2 D, left). This result was likely due to donor-specific CD8 T cell–mediated suppression.
because CD8-depleted PBMCs made equally strong DTH responses to both donor and autologous B-LCLs. Addition of flow-sorted HA-1A2-low CD8 T cells completely suppressed the DTH response to donor, but not autologous, B-LCLs. Similar numbers of tetramer-negative CD8 T cells failed to mediate significant suppression of DTH (Fig. 2 D, right).

Tetramer-sorted CD8+ T cells from patient I were placed in long-term culture with autologous HA-1H-pulsed DCs. Tetramer-low CTL lines expressed normal membrane levels of TCRαβ and CD8, but required a 100-fold higher HA-1H peptide level to sensitize autologous B-LCLs for lysis, as compared with tetramer-high CTL (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20031012/DC1).

Suppression of In Vitro IFN-γ Production by Addition of TR to Tε Cells. We questioned whether the HA-1A2-high T cells contained IFN-γ producers and whether the HA-1A2-low cells could suppress the IFN production. Tetramer-based flow sorting of PBMCs was performed and in vitro IFN-γ responses of the sorted cells were measured ELISPOT. In wells plated with 1.25 × 10^5 TDP as a source of APCs or with tetramer-sorted T cells alone (unpublished data), <5 IFN-γ spots/well were detected in the presence of 10 μg/ml of HA-1H peptide. When 10^5 HA-1A2-high CD8 T cells were stimulated with HA-1 peptide in the presence of APCs, substantial production of IFN-γ (Fig. 3, >200 spots/well) was observed. In contrast, 3 × 10^4 HA-1A2-low T cells failed to produce IFN-γ upon HA-1 peptide-specific stimulation. The same Tε cells completely inhibited HA-1-specific IFN-γ production by cocultured HA-1A2-high CD8 T cells (Fig. 3). This in vitro inhibition effect, unlike the suppression of the in vivo DTH response, was largely resistant to antibodies neutralizing TGFβ or IL-10 (10% recovery of response), but was sensitive to CTLA-4 antibody blockade (Fig. 3, 70% recovery). Anti-CTLA-4 blocking antibody could also reverse DTH unresponsiveness of whole PBMCs to HA-1H (unpublished data), indicating an important role of CTLA-4 in suppressor cell function both in vivo and in vitro.
HA-1H–specific T Cells Are Predominantly of the Effector Memory Phenotype. Further phenotype analysis of the HA-1–specific TR and TE cells is shown in Fig. S3 (available at http://www.jem.org/cgi/content/full/jem.20031012/DC1) and Table I. Although the tetramer-negative CD8+ T cells from the tolerant patient were predominantly CD28+, 78% of the TR cells and 68% of the TE cells were CD28+. The TR cells consisted mainly of CD45RO−CD62L−CCR7−T cells of the memory effector type (20). The tetramer-high CD8+ TE subset did contain a significant component of central memory T cells (Table I, 27%), but like the TR cells, were predominantly memory effector type.

Microchimerism as a Possible Source of Ongoing HA-1 Antigen Stimulation. We hypothesized that a source of ongoing antigen stimulation in the HA-1–regulated patients would account for the HA-1H–specific CD8+ T cells that regulate the DTH response. To identify a continuous source of HA-1 antigen in vivo, we tested for HA-1H microchimerism. DNA extracted from the PBMCs of patients I and II were analyzed for the presence of HA-1H genomic DNA. Microchimerism corresponding to the lower limit of detection of a PCR/SSP assay (a donor cell frequency of 1/104) was confirmed by sequence-specific oligonucleotide probe in patient I (Fig. 4 A). Next, a nested PCR assay using genomic DNA from flow-sorted T cells, B cells, monocytes, DCs (CD11c+ plus CD123+), and from in vitro–cultured monocyte-derived DCs was performed (Fig. 4 B). The strongest positive signals for HA-1H genomic DNA were detected in the DC-enriched subsets of both patients, despite the low cell recovery in this fraction. A weak positive signal was also detected in T cells and in cultured DCs from tolerant patient I. The HA-1H DNA signals in B cell and monocyte subsets were below the level of detection (1 in 105 cells) in both patients.

Table I. Phenotyping of Tetramer HA-1A2 Low, High, and Negative CD8+ T Cells by Flow Cytometric Analysis

| HA-1A2 subsets | CD8 T cell subset | CD45RO+ CD8 T cells |
|----------------|-------------------|---------------------|
|                | CTLA-4+ | CD28+ | CD45RO+ | CD62L+ | CCR-7+ | CCR-7− |
| Low            | 77      | 78    | 96      | 92     | 4      | 96     |
| High           | 50      | 68    | 90      | 82     | 27     | 73     |
| Negative       | 24      | 24    | 49      | 92     | 1      | 99     |

Cell surface expression was analyzed for all markers except CTLA-4 (intracellular). Data are summarized from the flow histograms (Fig. S3). Values shown are representative of two separate experiments.
provide an explanation for the retention of strong but tightly regulated T cell memory responses to the donor minor H antigen, HA-1. Coexistence of TGFβ-producing CD8+ T<sub>R</sub> cells with IFN-γ-producing CD8+ T<sub>E</sub> cells, each specific for different HIV epitopes, has been described in chronic HIV infection (21). A similar homeostatic equilibrium, involving CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells and CD4<sup>+</sup>CD25<sup>−</sup> T<sub>E</sub> cells has been described for the chronic phase of *Leishmania major* infection in mice (4).

The in vivo expansion of antigen-specific memory CD8<sup>+</sup> T cells with low binding to cognate peptide/MHC ligand and regulatory function is a novel finding. Chronic low avidity engagement of TCR with agonist peptide/MHC complexes in vivo has been shown to render memory CD4<sup>+</sup> T cells anergic (22). Indeed, we found that HA-1<sup>A2-low</sup> CD8<sup>+</sup> T cells proliferated poorly in response to HA-1 peptide-pulsed autologous DC, IL-7, and IL-2 stimulation in vitro. In contrast, HA-1<sup>A2-high</sup> CD8<sup>+</sup> cytotoxic T<sub>E</sub> cell lines showed excellent in vitro growth (unpublished data). These results argue that the T<sub>R</sub> cells are anergic memory effector T cells. Why antigen restimulation in vivo does not give a selective advantage to high avidity T<sub>E</sub> cells is not clear. CTLA-4 has been shown to attenuate strong signals generated through the TCR, while permitting the generation of weaker TCR signals (23). This regulatory role of CTLA-4 at the immune synapse has been proposed as a means of broadening the TCR repertoire recruited in response to antigen stimulation by limiting the selective advantage of high affinity over low affinity TCR<sup>+</sup> clones (24). Indeed, the majority (>75%) of the HA-1<sup>A2-low</sup> (T<sub>R</sub>) cells, and half of the T<sub>E</sub> cells coexpressed CTLA-4 (Table I and Fig. S3). Because anti-CTLA-4 blocking antibody could reverse T<sub>R</sub> cell-mediated suppression both in vivo and in vitro, we propose that CTLA-4 plays a dual role in HA-1<sup>H</sup>-specific CD8<sup>+</sup> cell responses, favoring growth and function of T<sub>R</sub> cells, while restraining that of T<sub>E</sub> cells.

As yet, no MHC class II-restricted T cell epitopes encoded by the HA-1 gene have been defined. Thus, we cannot rule out the possible involvement of HA-1–specific CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells at some phase of the host regulatory T cell response. The fact that CD8<sup>+</sup> T cell–depleted PBMCs of the tolerant patient failed to manifest linked suppression of DTH to donor B-LCLs would suggest that CD4<sup>+</sup> T<sub>R</sub> cells were not required. However, it is clear that CD4<sup>+</sup> T<sub>R</sub> cells do mediate regulation of DTH and skin allograft rejection in MHC-mismatched, tolerant renal allograft recipients (6, 25, 26).

The hematopoietic-specific HA-1 antigen is not expressed by kidney parenchymal cells. This suggests an indirect role for HA-1–specific T<sub>R</sub> cells in maintaining renal transplant tolerance. All three HA-1–mismatched patients displayed linked suppression of DTH responses to a third party antigen when HA-1<sup>H</sup> was present (Fig. 1 B). Therefore, we speculate that the colocalization of HA-1–specific T<sub>R</sub> cells with donor-derived DCs (Fig. 4 B) may propagate infectious tolerance to antigens shed by the kidney parenchyma and presented by the same DCs to other minor H–specific T cells. The fact that low numbers of HA-1–specific CD8<sup>+</sup> T<sub>R</sub> cells could mediate linked suppression when donor leukocytes were used as the source of endogenous antigen (Fig. 2 D) supports this speculation. Although we cannot rule out the “null” hypothesis (i.e., that HA-1 microchimerism in blood DC precursors is epiphenomenal), we also cannot exclude the alternative possibility that microchimerism sustains minor H antigen–specific CD8<sup>+</sup> T memory cells as has been suggested recently (27). Based on the HA-1 typing of patient I’s daughter born 20 yr before transplant, as well as the deduced HA-1 heterozygosity of her parents, either the transplant donor (H/H), maternal (H/R), or fetal (H/R) exposures may have contributed to her HA-1 microchimerism.

If the HA-1<sup>H</sup> DCs do contact the recipient’s HA-1–specific T cells in vivo, it is noteworthy that the CD8<sup>+</sup> T<sub>R</sub> cells were found in the small lymphocyte population (Fig. 2 A), and thus by scatter profile were not typical of effector memory T cells that have recently encountered antigen. In this regard, the encounter of plasmacytoid DC with allogenic CD8<sup>+</sup> T cells has been found to induce an IL-10–producing T<sub>R</sub> cell with a smaller size (based on forward scatter) than that of antigen-activated T<sub>E</sub> cells (28).

In conclusion, our findings indicate that lifelong peripheral tolerance to an organ allograft can be achieved without the loss of immunologic memory to donor antigen. If these results can be confirmed in other tolerant transplant recipients, it suggests a new rationale for clinical tolerance strategies, taking advantage of the inhibition of memory CD8 T<sub>E</sub> cells by regulatory T cells with low avidity for the same cognate antigen.

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