Intracellular MHC Class II Controls Regulatory Tolerance to Allogeneic Transplants

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*J Immunol* published online 25 January 2010
http://www.jimmunol.org/content/early/2010/01/25/jimmunol.0803664
Intracellular MHC Class II Controls Regulatory Tolerance to Allogeneic Transplants

Christian LeGuern,* Yoshinobu Akiyama,† Sharon Germana,* Katsunori Tanaka,† Luis Fernandez,* Yoshiko Iwamoto,† Stuart Houser,‡ and Gilles Benichou†

MHC class II (MHCII) genes have been implicated in the regulation of T lymphocyte responses. However, the mechanism of MHCII-driven regulation remains unknown. Matching for MHCII between donors and recipients of allografts favors regulatory T cell tolerance to transplants and provides a unique opportunity to study this regulation. In this study, we investigated MHCII regulation using transfer of donor MHCII genes in recipients of cardiac allografts. Transfer of MHCII IA<sup>b</sup> genes in the bone marrow of CBA mice (H-2<sup>b</sup>) prior to the grafting of IA<sup>b</sup> fully allogeneic C57BL/6 (B6, H-2<sup>b</sup>) heart transplants resulted in donor-specific tolerance associated with long-term survival of B6, but not third-party, allografts without sustained immunosuppression. Strikingly, the majority of accepted heart transplants (>170 d) were devoid of allograft vasculopathy. Further studies indicated that intracellular IA<sup>b</sup> initiated the tolerogenic process, which was mediated by regulatory T cells (Tregs) that polarized antigraft responses to Th2 cytokine producers. This mechanism seems to be unique to MHCII genes, because previous MHC class I gene-based therapies failed to produce Tregs. These results demonstrate the key role of MHCII in the induction of Tregs. They also underscore a potential mechanism of specific inactivation of T cells in this model; when activated by IA<sup>b</sup> grafts, IA<sup>b</sup>-specific Tregs repress the entire allosresponse to C57BL/6 transplants (including MHC I and minor Ags), thus mediating T cell tolerance.

The Journal of Immunology, 2010, 184: 000–000.

Major histocompatibility complex class II (MHCII) glycoproteins are known to play a deciding role in the selection of helper and regulatory CD4<sup>+</sup> T lymphocytes (1). The contribution of MHCII molecules plus self-peptide (pMHCII) complexes to CD4<sup>+</sup> T cell differentiation/activation has been well documented (2). Likewise, pMHCII complexes are involved in the thymic differentiation of naturally occurring CD4<sup>+</sup> CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) (3, 4). In addition to their prominent role in CD4<sup>+</sup> cell differentiation, MHCII molecules have been implicated in the regulation of immune responses, a function that was the cornerstone of their discovery (5) but remains poorly understood. It is widely accepted that the avidity of MHCII heterodimers for foreign peptides and the density of resulting pMHCII complexes on APCs modulate CD4<sup>+</sup> T cell activation (6–9). However, there is mounting evidence that the regulatory function of MHCII molecules is not confined to their ability to bind or not bind peptides. Indeed, inverse correlations between peptide avidity for MHCII and the magnitude of T cell responses were reported (10, 11). Other instances in clinical and experimental transplantation showed that matching for MHCII loci between graft donors and hosts downmodulated T cell responses to allografts and improved graft survival (12, 13). These data indicated that immune regulation via MHCII was mostly independent of the avidity of MHCII for graft-derived peptides and that transplantation models may be especially suited to decipher the regulatory function of MHCII genes.

To assess whether MHCII regulatory control on T cell responses was mediated by MHCII genes and not by other genes mapping in the MHCII locus, pilot gene-therapy experiments were carried out in a preclinical transplantation model (14). The results demonstrated that transfer of donor MHCII genes into bone marrow cells (BMCs) of future graft recipients induced immune tolerance to kidney transplants that were fully allogeneic to the hosts (15, 16). They also suggested that the MHCII-induced tolerance was regulatory, because T cell responses to all donor major and minor Ags were repressed. However, the mechanism of downregulation of immune responses by MHCII remains to be demonstrated.

The present study investigated the mechanism of MHCII control over T cell responses to allogeneic cardiac transplants in mice. We demonstrated that transfer of a single donor MHCII gene in recipient BMcs resulted in donor-specific tolerance and long-term survival of fully allogeneic transplants expressing this donor MHCII gene. MHCII-induced tolerance was selectively carried by Tregs and involved cytosolic forms of transferred MHCII molecules expressed in/on APCs. Furthermore, MHCII gene transfer prevented the onset of chronic rejection characterized by cardiac allograft vasculopathy (CAV), the chief cause of graft loss in clinical transplantation (17). These findings provide a novel mechanism to account for the regulatory role of MHCII genes, by showing that MHCII has the unique property of inducing Tregs that ultimately suppress the T cell allosresponses involved in acute and chronic allograft rejection.
**Materials and Methods**

**Animals**

Six- to eight-week-old female BALB/c (H-2b), CBA (H-2b), and C57BL/6 (B6; H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained in our pathogen-free facility at the Massachusetts General Hospital animal facility and treated according to institutional guidelines.

**Cells**

Dendritic cells (DCs) were derived from retrovirally transduced BMCs from CBA/J mice. Nonadherent transduced BMCs (2 × 10^6 cells/ml) were plated in 4 ml RPMI 1640 medium containing 500 U/ml recombinant mouse GM-CSF (BioSource International, Camarillo, CA) in six-well plates. Every other day, one-half of the medium was refreshed. On day 7, 50 U/ml recombinant murine IL-4 (BioSource International) was added in addition to GM-CSF to derive mature DCs (mDCs). Cells were harvested for analyses 4 days later. CD11c^+^ cells for quantitative RT-PCR (qRT-PCR) analysis were purified over magnetic columns after staining with biotin-conjugated anti-CD11c mAb (clone HL3, BD Pharmingen, San Diego, CA), followed by anti-Biotin MicroBeads (Miltenyi Biotec, Auburn, CA). CD4^+^ T cell subset enrichment (>90%) was performed on spleenocytes using the Regulatory T Cell Isolation Kit (Miltenyi Biotec).

**Retroviral vectors**

Constructs contain the L.n vector backbone, modified with a myeloproliferative sarcoma virus enhancer in the 3′ long terminal repeat (19). The IAb.RV construct derived from the plasmid pBMB19 containing the porcine MHCIi DRA and DRB cDNA, spaced by the internal ribosome entry site (IRES), from the 5′ untranslated IGH-binding protein (19). The following modifications were made: the EMC-neo(-)SV40 ori fragment was removed by digestion with BglII and ClaI, and the SLA-DRB and SLA-DRA sequences were replaced, respectively, with murine sequences for I-A^b^ (bases −1682 to 900-bp EcoRI X Hinfl II fragment from plasmid pCEX-AB^b^) and I-A^k^ (bases −77 to 901, 985-bp EcoRI fragment from plasmid pCEX-A^k^), kindly provided by R.N. Germain, National Institutes of Health, National Institute of Allergy and Infectious Diseases (Bethesda, MD).

To construct the IAb-GFP.RV plasmid, the stop codon of the I-A^k^ cDNA in IAb.RV was mutated (in bold, underlined), and a 3′ AaeI restriction site was added using the QuikChange Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) and primers 5′-CCAGAGCCTTCAAGTGACACCCGGTG- AAAAAAGGACGCCG-3′ and 5′-CGCCTCCTCTTACGGGTTGACCTT- AAGGGCCCTGCGG-3′. The enhanced GFP (eGFP) cDNA, isolated from plasmid pEGFP-N1 (BD Clontech, Palo Alto, CA), was cloned in-frame with the I-A^k^ mutated cDNA. The GFP.RV construct (pBM25.1) was previously reported, and virus-containing supernatants were prepared as described (20).

**Retroviral transduction**

CBA/J mice were treated with fluorouracil (150 mg/kg, i.v.), and BMCs were harvested 7 days later for transduction. Transduction was performed as described (20), with some modifications: recombinant mouse stem cell factor was from BioTransline International, and retrovirus-coated wells were preloaded with retrovirus for 3 h at 37°C. L cell fibroblasts were transduced using supernatants diluted 1:5 in complete DMEM + 16 μg/ml polybrene for 2 h at 37°C.

**Conditioning protocols for bone marrow and heart transplantation**

Transplantation of transduced BMCs (1 × 10^6 cells/mouse, i.v.) was performed in CBA mice receiving two injections of anti-CD4 (GR1.5), anti-CD8 (2.43) Abs (1 mg each/mouse, i.p.) at days −3 and −1 relative to bone marrow transplantation. Animals also received 10 mg/kg Busuliflex at days −4, −3, −2, and −1 (21). Eight weeks later, animals received B6 or BALB/c heart grafts together with 1 mg anti-CD8 i.p. (22). Transplants were monitored daily by palpation in a blinded fashion. Undetectable heart impulses for two consecutive days was considered rejection. Statistical analysis was performed using the Student t test. The prevalence and severity of CA were quantified by a morphometric approach (23). Briefly, a minimum of 50 vessels were examined per specimen using elastic-stained tissue sections. Severity of CA was quantified as the percent stenoses of involved vessels (intimal area + intimal area + luminal area) × 10^3 and is reported as the mean ± SE stenosis of all vessels with CA. For adoptive transfers, long-term tolerant and naïve CBA mice received donor-specific transplants (DSTs; 250 μl blood from B6 mice/mouse) 3 and 2 wk prior to cell transfer. Purified CD4^+^CD25^−^ or CD4^+^CD25^+^ T cells from tolerant or naïve CBA mice were infused i.v. into naïve CBA mice at day 0, along with 1 mg/mouse anti-CD8 Ab. Mice were then transplanted with B6 hearts.

**RT-PCR and qRT-PCR**

RNA was isolated from cells and tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was prepared from 5 μg DNase-treated RNA using oligo(dT) primers and the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). One-fourth of the total cDNA was used as template for each PCR reaction. Primers were 5′-AAGATGTTGAG- CGCCATCG (I-A^b^) and 5′-CCAGAGCCTTCTTCTTCTT (I-A^k^) for I-A^b^ proviral sequences; 5′-CAGCATCTTCTTCAAGGAG and 5′- GTGTTCTGGTGTGTTGCTG-3′ for eGFP; and 5′-GGAATTCTGCGA- AAAWGRTGARGACG and 5′-GGATCCTCTGCTGTGACGTYG- TGTGC for detection of CD2. Reaction conditions for I-A^b^ and eGFP sequences were 1 × Buffer A (Fisher Scientific), 200 μM each 2′-deoxy- nucleoside 5′-triphosphate, 0.5 μM primer, and 2.5 U Taq polymerase (Fisher Scientific) in 50 μl. Cycle conditions were 94°C/30 s, 55°C/30 s, and 72°C/1 min. Ex- pression levels were normalized to hypoxanthine phosphoribosyltransferase. For hypoxanthine phosphoribosyltransferase, we used 5′-AGACGACTTCTTCAAGTCCG and 5′-AGCACGACTTCTTCAAGGAG and 5′-AGCACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG and 5′-AGACGACTTCTTCAAGGAG

**ELISPOT assays**

These assays were performed according to published techniques (25).

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**FIGURE 1.** Conditioning protocol for I-A^k^ gene therapy. CBA (I-A^k^, I-E^k^) recipients were conditioned with anti-CD4, anti-CD8 mAbs (days −3 and −1) and Busuliflex (days −4, −3, −2, and −1) prior to transplantation of syngeneic BMCs that were transduced with the IAb.RV or GFP.RV vector (Fig. 2A). Eight weeks after BM transplantation, animals received B6 hearts (I-A^k^, I-E^k^) with anti-CD8. For adoptive transfers, tolerant (P0D 170) and naïve CBA mice received DST at days −21 and −14 relative to the adoptive transfer. Purified CD4^+^CD25^−^ or CD4^+^CD25^+^ T cells from tolerant or naïve CBA mice were infused at day 0 into naïve CBA recipients, together with 1 mg of anti-CD8. Animals were transplanted with B6 cardiac allografts the following day.
Results

Expression of a single-donor MHCII gene prevents rejection of fully allogeneic heart transplants

As depicted in Fig. 1, CBA (H-2k) mice were reconstituted with syngeneic BMCs that were previously transduced with a vector containing the allogenic MHCII Aβ gene (MHCII IAb.RV) or a control GFP gene (GFP.RV) (Fig. 2A). Hereafter, these mice are referred to as IAb-CBA and GFP-CBA mice, respectively. These animals were monitored for repopulation of blood cells and showed normal levels by 8 wk after bone marrow (BM) infusion compared with naive CBA animals (data not shown). At that time, IAb-CBA mice received one injection of anti-CD8 mAb and were transplanted with fully allogeneic, but IAβ-matched, B6 (H-2b) or third-party BALB/c (H-2d) hearts. Control animals included GFP-CBA mice injected with anti-CD8 and transplanted with B6 hearts. Representative histology of two long-term accepted B6 hearts from IAb-CBA mice (C, D) and a B6 transplant rejected by a control GFP-CBA mouse (E) (Verhoeff elastic stain, original magnifications ×200 (C), ×320 (D), and ×640 (E) at POD 177, 175, and 23, respectively). CAV lesions were quantified as detailed in Materials and Methods.

FIGURE 2. Survival of IAβ+ cardiac transplants in IAb-CBA and GFP-CBA recipient mice. A, The IAb-RV vector contained cDNA for the IAαβ- and IAββ-chains, spaced by an IRES. Transcription was under long terminal repeat promoter control (arrow). Intact vector transcripts were detected by RT-PCR (double arrowheads). The IAb-GFP.RV vector was constructed on the same backbone with the addition of the GFP sequence that was fused, in frame, to that of I-Aαβ. The GFP.RV vector included the GFP sequence. B, IAαβ-transduced CBA mice (n = 15) received B6 (n = 11) or third-party BALB/c hearts (n = 4) that were monitored for survival. Controls were CBA mice (n = 7) engineered with the GFP.RV vector and transplanted with B6 hearts. Representative histology of two long-term accepted B6 hearts from IAb-CBA mice (C, D) and a B6 transplant rejected by a control GFP-CBA mouse (E) (Verhoeff elastic stain, original magnifications ×200 (C), ×320 (D), and ×640 (E) at POD 177, 175, and 23, respectively). CAV lesions were quantified as detailed in Materials and Methods.

FIGURE 3. T cell alloresponses in IAb-CBA mice. IL-2 (A, D), IFN-γ (B, E), and IL-4 (C, F) ELISPOT assays were performed on splenocytes from naive CBA (white bars) and IAb-CBA (black bars) mice. Assays were done 8 wk after BM transplantation, prior to heart transplantation (n = 3) and 10 d after transplantation of B6 heart grafts (n = 3). Stimulator cells were irradiated splenocytes from BALB/c or B6 mice. MEM: control medium. Results are mean ± SD.

before transplantation

B6

MEM

after transplantation

B6

MEM

IL-2

γIFN

IL-4

Spots per million T cells
(MST, 18 ± 7 d; n = 15; data not shown). Likewise, GFP-CBA recipients acutely rejected B6 heart transplants (MST, 29 ± 6 d; Fig. 2B). In contrast, the vast majority of B6 cardiac allografts, placed in IAb-CBA recipients, survived indefinitely (>150 d). A second series of IAb-CBA mice transplanted with a third-party BALB/c (H-2d) heart acutely rejected their grafts within 19 d posttransplantation (Fig. 2B). Thus, the transfer of a single donor MHCII IAβ gene in recipient BMCs was sufficient to prevent the acute rejection of IAβ grafts that were fully allogeneic with regard to MHC class I and minor Ags to the host genotype. T cell tolerance induced by MHCII gene therapy seemed to be donor-specific, because third-party allografts were promptly rejected.

**MHCII gene transfer markedly reduces chronic rejection characterized by CAV**

Chronic rejection characterized by CAV is the main cause of heart graft failure after the first year of transplantation in patients. In this study, we investigated whether long-term surviving B6 hearts from IAb-CBA recipients exhibited CAV lesions. The onset and severity of CAV were analyzed using our standard histomorphometric test to evaluate intimal thickening and subsequent luminal occlusion on numerous graft vessels (23). The B6 heart grafts being rejected by control GFP-CBA mice (Fig. 2B) displayed severe CAV associated with fibrosis and intimal occlusion that was detectable as early as 14–28 d posttransplantation (Fig. 2E). In contrast, the B6 hearts collected from IAb-CBA mice had no signs of chronic rejection and displayed normal vessel architecture for as long as 177 d after transplantation (Fig. 2C, 2D). Therefore, in addition to preventing acute allograft rejection, expression of donor-type MHCII gene in hematopoietic cells of graft recipients also thwarted chronic rejection of heart allografts.

**Expression of donor MHCII in graft recipients modulates antidonor alloresponses**

Acute rejection of allotransplants is dependent upon the activation/expansion of proinflammatory T cells secreting type 1 cytokines (IL-2 and IFN-γ) (26). Conversely, T cells secreting type 2 cytokines (IL-4 and -10) have been implicated in the prolongation of allograft survival (27), presumably by suppressing inflammatory T cell immunity (28). These observations prompted us to examine whether the IAβ gene transfer in B6A mice altered their ability to mount a T cell-mediated alloresponse to B6 allogeneic cells (Fig. 3). The frequency and specificity of activated T cells producing type 1 (IL-2 and IFN-γ) or type 2 (IL-4) cytokines were measured in control CBA and IAb-CBA mice. To test this, T cells from control CBA and IAb-CBA mice were collected prior to and 10 d after placement of a B6 cardiac allotransplant. These cells were then cultured for 24–48 h in the presence of donor B6 or third-party BALB/c irradiated stimulators and the numbers of types 1 and 2 cytokine-producing T cells were assessed using an ELISPOT assay. A similar frequency of IL-2 producers (~120 spots/million T cells) was observed for CBA and IAb-CBA T cells responding to third-party BALB/c stimulators, irrespective of the presence of B6 allografts (Fig. 3A, 3D). A comparable trend developed in anti-BALB/c, IFN-γ responses of CBA and IAb-CBA mice (Fig. 3B, 3E, top). Thus, expression of IAβ in IAb-CBA mice did not affect the anti-third party (BALB/c H-2d) T cell response that, in an MLR, is mainly directed

![FIGURE 4. Time course analysis of IAα transcription in vivo. A. RT-PCR analysis of IAα and control CD2 gene transcription in tissues from long-term tolerant CBA #13 (POD 176). Proviral IAα sequences were detected by annealing to a [32P]-IRES–specific oligonucleotide. B. IAα transgene transcription in PBMCs, lymph nodes (LN), spleen, and thymus. Two rejector IAb-CBA (Rej; tested 11 wk after BMC infusion) and six long-term tolerant IAb-CBA (Tol; 30–32 wk) mice were monitored by RT-PCR for proviral IAα transcription. *Number of animals positive for IAα/number tested. Rej, rejector IAb-CBA mouse; Tol, long-term tolerant IAb-CBA mouse.](http://www.jimmunol.org/)

![FIGURE 5. Patterns of IAα transgene expression in APCs. A. BM Scalc+ progenitors from CBA mice transduced with the empty (mock) or IAb.RV (IAb) vectors were analyzed 48 h after transduction for IAα expression. B. Subsets of DCs, derived from BMCs from CBA mice, were sorted as iDCs (CD11c+IAbdCD86+) or mDCs (CD11c+IAbαCD86−). Each subset was transduced with the GFP.RV vector and analyzed 5 d later for GFP expression. Data presented are from one of two experiments. C. I.: Unsorted DC cultures from CBA mice were transduced with the IAb.GFP.RV vector and tested for surface expression of recipient (IEk, dotted line) and vector-derived MHCII (IAb, black line) 7 d posttransduction. Shaded curve represents Ig isotype control. 2, 3: GFP expression in the iDC and mDC subsets that were derived from the same bulk DC culture transduced with the IAb.GFP.RV vector. Representative results from one of three experiments. 4, 5: DCs and L cells transduced with IAb.GFP.RV were analyzed for expression of the IAb-GFP fusion protein 1 wk after transduction. Similar data were obtained in four additional experiments using DC or other APC lines. 6: Real-time RT-PCR analysis of GFP transcripts in transduced DC and L cells from panels 4 and 5. 7: The IAbα IEβ+ lymphoblastic cell line A20 was transduced with the IAb.GFP.RV and tested 4 d later for endogenous (IAb), fine dotted line) and ectopic (IAb, bold dotted line) expression. Shaded curve represents staining with isotype control (Cir). 8: A20 cells from panel C7 were also tested for GFP expression in the absence (−) or presence (+) of monensin.](http://www.jimmunol.org/)
to allogeneic MHCII Ags (A<sup>a</sup> and E<sup>b</sup>) (29). We next measured the inflammatory (type 1) alloresponse of CBA and IAb-CBA mice to B6 stimulators. As seen in Fig. 3A and 3B, these mice developed similar Th1 responses (~280 spots/million T cells) prior to transplantation, suggesting that the primary anti-MHCII IA<sup>b</sup> response was intact in IAb-CBA mice. In contrast, the type 1 inflammatory alloresponse was abrogated in the IAb-CBA mice transplanted with a B6 heart compared with the control allotransplanted CBA recipients (Fig. 3D, 3E). In turn, high frequencies of activated donor-specific T cells secreting type 2 cytokine T cells were found in allotransplanted IAb-CBA mice (Fig. 3F). Therefore, the acceptance of IAb<sup>b</sup> B6 transplants in IAb-CBA mice is associated with a shifting from a type 1 proinflammatory antidonor T cell response toward an alloresponse dominated by T cells secreting type 2 cytokines.

**Transferred IA<sup>b</sup>** is expressed in lymphoid tissues and in BM-derived DCs

Eight weeks after transfusion of transduced BMCs, the IAb-CBA mice received a B6 heart under anti-CD8 therapy (Fig. 1). The presence of IA<sup>b</sup> transcripts was detected in PBMCs from 100% of animals 2 wk after BMC injection, whereas 77% (14/18) were positive at the time of heart transplantation (Fig. 4B). The IA<sup>b</sup> signal faded out over time in all IA<sup>b</sup>-treated animals to become undetectable in PBMCs by 18 wk posttransplant (Fig. 4B). A similar pattern of GFP expression was observed when the control GFP.RV vector was used for transduction. In addition, PBLs from control GFP-CBA mice were RT-PCR negative for IA<sup>b</sup> transcripts (Fig. 4A). Expression of IA<sup>b</sup> was further analyzed in lymphoid tissues from CBA-IAb mice that were collected at the time of allograft rejection (2/11 mice) or at postoperative days (PODs) 150–177 in six long-term tolerant animals (Fig. 4B).

To investigate IA<sup>b</sup> surface expression in transduced BMCs, we examined cells collected 48 h after ex vivo transduction. Surprisingly, IAb-RV-transduced BMCs were negative for surface IA<sup>b</sup> (Fig. 5A), whereas 10–15% of cells stained with specific Abs for endogenous IA<sup>b</sup> (data not shown). To ascertain whether the absence of surface IA<sup>b</sup> was due to poor transduction or to cell type-specific constraints, IA<sup>b</sup> expression was further studied in DCs derived from transduced BM, because DCs are key players in thymocyte negative selection (2). Bulk cultures of DCs derived from control GFP transduction were fluorescent, as were immature DCs (iDCs) and mDCs that differentiated from those cultures (Fig. 5B). Unexpectedly, iDCs and mDCs obtained from superinfected IAb-GFP BMCs were negative for IAb-GFP and positive for endogenous IA<sup>b</sup> (Fig. 5C, panels I–3). This suggests a defective IA<sup>b</sup> gene transcription in DCs and/or an intracellular cleavage of the ectopic IA<sup>b</sup> α- and β-chains. The former hypothesis was tested by comparing GFP transcript and protein levels in IAb-GFP–transduced DCs and control fibroblast cells (L cells). Results presented in Fig. 5C, panels 4–6, indicated that transcription of IAb-GFP in DCs was not compromised. Finally, monensin was used to block protein transport in A20 monocytic, MHCII<sup>b</sup> cells transduced with IAb-GFP.RV. Results suggested that cytosolic IAb-GFP fusion protein was short-lived (Fig. 5C, panels 7 and 8).

**Tregs from tolerant IAb gene recipients transfer transplantation tolerance to naive animals**

Our data on indefinite survival of fully allogeneic B6 heart grafts implanted in IAb-CBA recipients (Fig. 2B) lend support to the notion of complete inactivation of type 1 inflammatory alloresponses to B6 graft major and minor Ags, a phenomenon tentatively attributed to Tregs (30). Therefore, we examined whether acceptance of B6 allogeneic hearts could be achieved in naive CBA mice, recipients of purified Tregs from long-term tolerant IAb-CBA mice. The in vitro suppressive function of IAb-CBA Tregs was first assessed on two tolerant mice at 177 and 178 d post-heart transplantation by a standard Treg suppression assay (31). Results indicated that CD4<sup>+</sup>CD25<sup>+</sup> Tregs from IAb-CBA and naive CBA mice were poorly and equally suppressive of CD4<sup>+</sup>CD25<sup>+</sup> T cell proliferation (data not shown). To activate recipient Tregs in vivo, tolerant IAb-CBA mice (>160 d post-transplantation) were infused with two consecutive donor-specific blood transfusions (32). Two weeks later, the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs were monitored. Each point represents survival data from a single mouse. *Survival time (days; mean ± SD)*.

![FIGURE 6.](http://www.jimmunol.org/) Tregs from tolerant IAb gene recipients transfer transplantation tolerance to naive animals.
Treg and CD4⁺CD25⁻Foxp3⁻ effectors subsets were isolated (purity >90%) from DST-treated IAb-CBA recipients as well as from naive CBA mice injected or not with DST (Fig. 1). These cell populations were injected separately into naive CBA mice together with one dose of anti-CD8. Treated animals were challenged the following day with B6 allografts, according to the protocol detailed in Fig. 1. Transfer of CD25⁺ or CD25⁻ T cell subsets, isolated from untreated CBA mice, did not result in statistically significant improvements in graft survival over that of non-transferred controls (Fig. 6, left panel; 33 ± 3 d; p = 0.2). CBA mice that received CD25⁺ or CD25⁻ T cells collected from mice treated with DST only experienced accelerated rejection of B6 hearts (Fig. 6, middle panel; 16 ± 2 d; p = 0.01). More importantly, four of five naive CBA mice that were injected with purified CD25⁺ Tregs from tolerant IAb-CBA mice showed significant prolongation of graft survival (Fig. 6, right panel; p = 0.03). In contrast, three of four recipients of CD25⁻ cells from tolerant IAb-CBA mice rejected their transplants in the same time course as untreated CBA controls. These data underscored the potent role of tolerant Tregs at transferring tolerance, because a single dose of Tregs fostered significant prolongation of graft survival in immunocompetent recipients. Fig. 7 tentatively presents a model of Treg control of alloresponses, which will be discussed in detail below.

Discussion
The present study demonstrated that mice expressing a single donor-type MHCII molecule at the time of transplantation of fully allogeneic heart grafts become tolerant to the whole donor/recipient antigenic disparity, a phenomenon referred to as linked or infectious tolerance (33). The tolerogenic effects of MHCII gene therapy is not unique to the murine heart transplantation model; we previously reported that MHCII gene transfer induced tolerance to MHCII transgene-matched renal allografts in large animals (16). Transfer of fibroblasts expressing donor MHC class I and MHCII prolonged cardiac allograft survival in mice (34), suggesting an equal role for MHCII and MHCII genes in regulating immune responses. However, in contrast to our gene-therapy approach, these experiments did not induce transplantation tolerance. In addition, retroviral transfer of MHCII K⁺ gene in BMCs also failed to induce spreading tolerance to K⁺⁺ grafts coexpressing third-party Ags (35). Thus, our findings strongly suggest that induction of spreading tolerance is a property of MHCII molecules. Although transduction rates were not as high as those for CBA BMCs, extended prolongation of graft survival was also observed in BALB/c mice receiving IAb genes and B6 transplants (75 ± 6 d; n = 10). This approach also established that linked tolerance was preferentially carried out by Tregs (Fig. 6). These points imply that the immune regulatory function of MHCII genes, which has been the cornerstone of their discovery (36), resides, in part, in their unique ability to generate Tregs.

The long-term surviving heart transplants (>170 d) presented no sign of chronic rejection, the major cause of late graft loss in clinical transplantation (Fig. 2) (37). Prevention of CAV development has been reported in other models using heavy conditioning with costimulatory blockade, cytolytic drugs, and donor BM infusions (38). Remarkably, our study showed that transduction of recipient BMCs with a single donor MHCII gene can achieve tolerance to fully allogeneic heart transplants, a result showing that activation of donor-specific Tregs can be sufficient to prevent CAV.

B6 heart transplants survived indefinitely in IAb⁻/⁻ mice, whereas third-party BALB/c cardiac allografts were acutely rejected. This supports a model of MHCII allele-specific tolerance involving the recognition of ectopic IAb heterodimers (direct recognition) and/or IAb⁻/⁻-derived peptides presented by recipient MHC H-2k molecules (indirect recognition). Testing for IAb sur-

face expression on transduced BMCs revealed no detectable IAb signal on whole BM or Sca1⁺cd41⁻ hematopoietic precursors, although IAb transcripts were consistently detected by RT-PCR. Likewise, blood cells from IAb⁻/⁻ mice exhibited no IAb on their surface but expressed IAb⁺ transcripts (Fig. 4). Analysis of IAb expression in DCs derived from IAb-transduced BMCs confirmed the absence of surface IAb molecules, despite levels of IAb transcripts that were compatible with surface expression (Fig. 5). Results from preliminary studies suggest that the α/β-chain pairing, rather than a limiting pool of the invariant chain, is a critical checkpoint controlling ectopic IAb⁺ chain cleavage. Because DCs are involved in the establishment of CD4⁺ T cell tolerance to self-Ags (39, 40), the absence of surface IAb molecules on IAb⁺-transduced DCs suggests that the presentation of IAb intracellular chains or IAb⁻/⁻-derived peptides by recipient MHC molecules underlies tolerance induction in this model. Additional data from our group support the MHCII peptide hypothesis. We observed that human B cell lines, transduced with a pig MHCII DR gene, did not express surface pig DR but stimulated autologous human T cell proliferation, which was only blocked by anti-human MHCII mAbs (41).

Immunocompetent CBA recipients were used to demonstrate acceptance of B6 allografts following the injection of Tregs from tolerant IAb-CBA mice (Fig. 6). Although other successful Treg/tolerance transfer protocols have been described using immunocompetent Rag⁻/⁻ recipients (42), to our knowledge, this is the first report on efficient transfer of graft acceptance in immunocompetent recipients via a single treatment with isolated Tregs. The initial in vivo priming to donor blood cells, and likely to cognate IAb, was potentially the cause of this favorable outcome, because injection of MHCII⁺ cells abolished the DST effect (43).

Treg TCR specificity was shown to be different from that of Th cells that they ultimately suppress (44). Our results suggest that MHCII allele-specific tolerance may be driven by the recognition of IAb peptides displayed on BM-derived APCs. This suggests that naturally occurring Tregs might differentiate in the thymus following recognition of MHCII/MHCII peptide complexes. We hypothesize that the transfer of the IAb⁺ gene in CBA mice promoted the development of IAb⁻/⁻ peptide-specific Tregs (Fig. 7; gene therapy [GT] Tregs). IAb⁻/⁻-treated animals would only accept IAb⁺ transplants because the latter provide IAb determinants via the direct or indirect pathways for secondary stimulation of GT Tregs and suppression of the antigraft responses. Tolerance to autografts tissues would proceed along the same mechanism involving, this time, native CBA Tregs. Data from autoimmune models have stressed the pivotal role played by MHCII-derived peptides in the reversal of defective Treg functions. For instance, the injection of NOD mice with self-IEB peptide led to Treg activation (45), whereas binding of IE-derived peptides on IA heterodimers prevented collagen-induced arthritis (46). The specificity of naturally occurring Tregs for self-MHCII determinants is consistent with results from studies that showed Treg thymic precursors differentiating in the medulla (3), a compartment that is known to selectively display MHCII peptide/MHCII complexes (47). Such bias of Treg specificity for MHCII determinants may explain how MHCII genes control the effectors as well as the regulatory phases of T lymphocyte responses.

Acknowledgments
The authors are grateful to Kaela Golstein for technical assistance and to Dr. Christene Huang for critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.
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