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The Male Minor Transplantation Antigen Preferentially Activates Recipient CD4+ T Cells through the Indirect Presentation Pathway In Vivo

Yifa Chen, Yilmaz Demir, Anna Valujskikh, and Peter S. Heeger

To evaluate the priming and trafficking of male Ag-reactive CD4+ T cells in vivo, we developed an adoptive transfer model, using Marilyn (Mar) TCR transgenic T cells that are specific for the H-Y minor transplantation Ag plus I-Aα. By manipulating donor and recipient strain combinations, we permitted the Mar CD4+ T cells to respond to the H-Y Ag after processing and presentation by recipient APCs (indirect pathway), or to the male Ag as expressed on donor APCs (direct pathway). Mar CD4+ T cells responding through the indirect pathway specifically proliferated and expressed activation markers between days 2 and 4 post-transplant, migrated to the graft 2–3 days before cessation of graft heartbeat, and were detected in close proximity to transplant-infiltrating recipient APCs. Intriguingly, adoptively transferred Mar T cells did not respond to male heart or skin grafts placed onto syngeneic MHC class II-deficient female recipients, demonstrating that activation of Mar T cell preferentially occurs through cognate interactions with processed male Ag expressed on recipient APCs. The data highlight the potency of indirect processing and presentation pathways in vivo and underscore the importance of indirectly primed CD4+ T cells as relevant participants in both the priming and effector phases of acute graft rejection.


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6 Abbreviations used in this paper: KO, knockout; GFP, green fluorescent protein; GIL, graft-infiltrating lymphocyte; Mar, Marilyn; WT, wild type; KO, knockout.
**Materials and Methods**

**Animals**

Male and female C57BL/6 (H-2b), C3H (H-2k), (C57BL/6 × C3H)F1 (H-2d), and green fluorescent protein (GFP) transgenic C57BL/6-TgN(ActbEGFP)10sb (H-2b, GFP transgenic) mice, age 6–8 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME). Congenic C57BL/6 MHC class II KO mice (I-A^b deficient) were purchased from Taconic Farms (Germantown, NY). Male and female TCR transgenic Marilyn RAG2 KO mice (H-2b, Mar), age 6–8 wk, were obtained as a generous gift from P. Matzinger (National Institutes of Health, Bethesda, MD) and O. Lantz (Institut National de la Sante et de la Recherche Medicale, Paris, France). All animals were maintained and bred in the pathogen-free animal facility at the Cleveland Clinic Foundation.

Intercrossing Mar mice, GFP transgenic mice, and their F1 progeny, and screening for GFP, the Mar transgene, and the absence of RAG by PCR allowed us to derive double-transgenic Mar^T^ GFP^R^ RAG KO animals. For genotyping, DNA was prepared from the ears at the time of weaning (3 wk of age) and analyzed by PCR for the presence of Mar transgene, as previously described (18). To screen for the presence of GFP transgene, animals were bled from the tail vein, and blood cells were examined with an immunofluorescent microscope.

**Peptides**

HYD69p (NAGENSNRANSSRSS) and chicken OVA323-339 (OVAp, KISQAIAHAAHEAEAG) were synthesized by Research Genetics (Huntsville, AL) at >90% purity.

**Placement and evaluation of skin and cardiac transplants**

Full-thickness skin grafts were placed, as customarily performed by our laboratory (4, 5). Bandages were removed on day 7, and the grafts were inspected daily. Rejection was defined as greater than 90% necrosis. Vascularted heterotopic cardiac allografts were placed in the abdomen, as described (20, 21), and palpated daily for evidence of a heartbeat. Rejection was defined as a loss of palpable heartbeat. Grafts were harvested at the time of rejection or at predetermined time points posttransplant.

**Cell labeling**

Mar^T^ GFP^+^ CD4^+^ T cells were isolated by negative selection using commercially available murine CD4 T cell isolation columns from R&D Systems (Minneapolis, MN), following the instructions supplied by the manufacturer. Resultant cells were washed in HBSS, counted, and labeled with PKH26 red fluorescent cell linker (Sigma-Aldrich, St. Louis, MO), as described by the manufacturer. A total of 10^–20^ × 10^5^ cells was incubated in 2 ml of diluent supplied by manufacturer with 2 μM PKH26 dye for 3–5 min at room temperature. During the incubation, the tube was periodically inverted to assure uniform labeling. The staining reaction was stopped by inverting the tube for 1 min at room temperature with alkaline phosphatase-conjugated anti-GFP Ab (Rockland, Gilbertsville, PA; 1/1000 dilution). After three additional PBS washes, the sections were incubated with biotin-conjugated streptavidin (stock concentration; DAKO) and developed using the Novared Substrate Kit (Vector Laboratories, Burlingame, CA). Sections were washed three times in PBS, and incubated for 90 min at room temperature with alkaline phosphatase-conjugated anti-GFP Ab (Rockland, Gilbertsville, PA; 1/1000 dilution). After three more washes with PBS, slides were developed using VECTOR Blue Alkaline Phosphatase Substrate Kit (Vector Laboratories). Sections were dehydrated with ethanol and mounted for analysis.

**ELISPOT assays**

Assays were performed, as previously outlined in detail (5). Briefly, ELISPOT plates (Millipore, Bedford, MA) were coated overnight with the capture Abs (obtained from BD Pharmingen) in sterile PBS, blocked with sterile 1% BSA in PBS, and washed three times with sterile PBS. Spleen cells (0.2–1 × 10^5^ per well) were plated in HL-1 medium (BioWhittaker, Walkersville, MD) with or without mitomycin C-treated stimulator cells (400,000 per well) and/or soluble Ags (HYD69p and OVAp at 0.1–10 μM) and then incubated at 37°C, 5% CO_2_ for 24 h. After washing with PBS, followed by PBS/0.025% Tween (PBST), detection Abs (obtained from BD Pharmingen) were added overnight. After washing with PBST, alkaline phosphatase-conjugated anti-biotin Ab (Vector Laboratories) diluted 1/2000 in PBST was added for 2 h at room temperature. The plates were developed, as previously described (5). The resulting spots were counted on an ImmunoSpot Series 1 Analyzer (Cellular Technologies, Cleveland, OH) (5).

**Isolation of organ-infiltrating lymphocytes**

Animals were anesthetized and injected i.v. with 10 ml sterile PBS until all organs were visibly blanched. Heart graft was individually harvested, cut into pieces with a sterile razor, and incubated with 25 mg of collagenase A (Boehringer Mannheim, Indianapolis, IN) in 25 ml of sterile HBSS at 37°C for 10 min with gentle intermittent gentle vortexing. Liver and lung tissues were similarly processed using collagenase V (Sigma-Aldrich). Resultant cells were filtered through a 40-μm cell strainer to remove larger pieces of residual tissue. RBCs were lysed from the filtrate, and the organ-infiltrating cells were stained with Abs to cell surface markers and analyzed by flow cytometry, as described above.

**Results**

A peptide determinant derived from the HYD69p gene is indirectly presented to recipient CD4^+^ T cells after heart transplantation

Previously published studies showed that placement of male B6 skin onto female B6 recipients primes recipient CD4^+^ T cells specific for an I-A^b^-restricted peptide determinant, HYD69p, derived from the Dby gene locus of the H-Y Ag (23). To establish a model system for studying indirectly primed CD4^+^ T cells following heart transplantation in vivo, we transplanted male or female C3H (H-2^k^) cardiac allografts into female B6 (H-2^k^) recipients. In this fully MHC-disparate strain combination, acute rejection results in
cessation of donor heartbeat within 8–12 days posttransplant. Consistent with work in an analogous skin graft model (19), we reasoned that transplantation of a male C3H heart into a female B6 recipient would result in indirect processing of the donor-derived male (H-Y) Ag, subsequent presentation in the context of recipient I-A<sup>b</sup>, and thereby would prime recipient HYDhp-specific CD4<sup>+</sup> T cells. As shown in Fig. 1, A and B, splenic immune cells obtained at the time of rejection from B6 recipients of male C3H heart transplants specifically responded to HYDhp (indirect priming) by producing IFN-γ and IL-2, but no IL-4 or IL-5 (data not shown), consistent with a type 1 cytokine profile. No cytokine production was detectable in response to a control I-A<sup>b</sup>-restricted peptide, OVA 323–339, confirming the specificity of the result. Moreover, no HYDhp-specific responses were detectable in female B6 recipients of female C3H hearts (no HY Ag in the graft) or in naive (nontransplanted) female B6 mice. Splenic immune cells from recipients of both male and female C3H heart transplants responded strongly to donor strain (C3H) stimulator cells, demonstrating T cell priming through the direct pathway as well.

Graft-infiltrating lymphocytes (GILs) were then isolated from allografts undergoing rejection on day 8–10 posttransplant and tested for recall responses to donor Ags. As shown in Fig. 1C, HYDhp-reactive T cells, as well as antidonor (C3H)-reactive T cells, were detectable within the C3H male allografts. The results confirm that HYDhp is a donor heart-derived determinant that is processed and presented to recipient CD4<sup>+</sup> T cells through the indirect pathway, and they further suggest that the indirectly primed T cells migrate to the donor organ.

**Indirect vs direct priming in vivo**

To more precisely study indirectly primed CD4<sup>+</sup> T cells following heart transplantation, we made use of a TCR transgenic mouse, Mar, backcrossed to B6 RAG KO in which all T cells are CD4<sup>+</sup> and are specific for HYDhp + I-A<sup>b</sup> (18). Notably, Mar T cells isolated from naïve Mar mice express a CD62L<sup>high</sup>/CD44<sup>low</sup> naive cell surface marker phenotype (Fig. 2A). In confirmation of published studies (19), Mar CD4<sup>+</sup> T cells do not cross-react with male C3H alloantigens (Fig. 2, B and C).

We next evaluated the kinetics of indirect priming to a cardiac allograft in vivo. We crossed the Mar mice with congenic B6 GFP transgenic mice so as to be able to readily identify Mar T cells adoptively transferred into WT recipients by their constitutive expression of GFP. Purified Mar GFP<sup>+</sup> CD4<sup>+</sup> T cells were then labeled with the red fluorescent membrane dye PKH26 and transferred into WT B6 female recipients of either male or female C3H heart grafts. Analogous to CFSE, PKH26 segregates equally among daughter cells such that in vivo proliferation can be assessed by flow cytometry using the amount of fluorescence detected on each cell.

Spleenic GFP<sup>+</sup> Mar T cells in recipients of C3H male heart grafts (primed through indirect pathway) proliferated (>4 cell divisions) and expanded significantly in number by day 6 posttransplant (Fig. 3 A, Table I). The percentage of GFP<sup>+</sup> Mar T cells increased from 0.36 ± 0.02% of the total spleen cells in control mice (grafted with C3H female hearts) on day 4–6 posttransplant, to 7.89 ± 2.15%...
To determine whether the addition of direct presentation by Ag expressed on donor APCs would affect the kinetics of priming (compared with indirect presentation), we next transplanted recipient B6 females with male (B6 × C3H)F1 heart grafts. Expression of the H-2k restriction elements on the donor graft theoretically permits direct presentation of HYDdbp + I-Ak complexes to recipient Mar T cells. The inclusion of the C3H alloantigens in the F1-transplanted heart is required to prime an alloreactive T cell repertoire capable of rejecting the graft so we can visualize the responding Mar T cells within the context of a normal alloimmune response (female B6 mice do not reject syngeneic male B6 heart grafts; data not shown). Interestingly, the inclusion of direct Ag presentation did not significantly accelerate the kinetics of Mar T cell priming (Fig. 5); multiple rounds of cell division and the induced expression of an activated cell surface phenotype occurred between days 2 and 4 posttransplant. Mar T cells did not divide when transferred into naive mice without transplants and they continued to express a naive cell surface phenotype.

To determine whether male grafts would prime Mar T cells in the absence of the indirect pathway, we transferred labeled Mar T cells into female B6 MHC class II KO recipients and performed transplantation with B6 male heart grafts. In this strain combination, the donor male Ag is expressed directly on donor APCs, but the absence of recipient MHC class II precludes processing and presentation via the indirect pathway. To our initial surprise, Mar T cells did not divide in MHC class II KO recipients of male B6 hearts; they did not expand in number, nor did they alter cell surface activation markers when compared with Mar T cells transferred into MHC class II KO controls that did not receive heart grafts (Fig. 6, Table I). In contrast to the results shown in Figs. 3–5, there was minimal proliferation, expansion, or activation of the Mar T cells on days 6 and 9 posttransplant. A small number of GFP+ Mar T cells divided in both the controls and the heart graft recipients, presumably representing background homeostatic proliferation. Identical results were noted using male (B6 × C3H)F1 heart grafts as donors (Table I).

Transferred Mar T cells remained detectable in nontransplanted B6 MHC class II KO females for >8 days, assures that the absence of recipient MHC II did not result in deletion or apoptosis of the transferred CD4+ T cells over this time period. Spleen cells from transplanted or control naive B6 MHC II KO recipients given GFP+ Mar T cells were isolated on day 9 posttransplant, cultured in vitro for 4 days with WT female B6 APCs plus HYDdbp (or OVA233–239 control), and then analyzed (% GFP+) by flow cytometry. HYDdbp stimulation specifically resulted in visual blástogenesis by day 2 and in expansion (0.25% GFP+ in HYDdbp-stimulated cultures vs 0.07% in controls) and activation (79.8% CD62Lhigh GFP+ Mar cells in controls vs 30% in HYDdbp-treated cultures, n = 2; data not shown) by day 4, confirming that the transfected T cells remained functional and capable of responding to their specific Ag. As an additional test of functionality, we placed PKH26-labeled GFP+ Mar T cells in B6 MHC class II KO and in control WT mice for 3 days. Spleen cells were then resolated and stimulated with additional WT B6 female splenic APCs (to provide a source of I-Ak) plus HYDdbp or OVA233–239 as a control (Fig. 7). Flow cytometry performed on culture day 7 clearly showed that the HYDdbp-stimulated Mar T cells isolated from both WT and MHC class II KO animals specifically lost expression of PKH26 (indicating cell division), underwent in vitro expansion, and expressed an activated cell surface phenotype (CD44high, CD62Llow). Thus, the lack of Mar responsiveness to donor B6 male heart grafts in MHC class II KO recipients cannot simply be attributed to an induced functional defect of the transferred Mar T cells in the MHC class II-deficient environment.

FIGURE 3. In vivo visualization of priming through the indirect pathway. A, PKH26-labeled CD4+ T cells from GFP transgenic Mar RAG KO mice were injected into B6 female recipients of male (left) or female (right) C3H heart grafts. Spleen cells were obtained 6 days after adoptive transfer. Histograms are gated on GFP+ cells. The results are representative of four animals per group studied at this time point. B, Kinetics and topography of activation. Representative two-color dot plots are shown for day 2 posttransplant (top) and day 4 posttransplant (bottom). Pooled peripheral lymph nodes are shown on the left, and spleen cells are shown on the right. The results are fully representative of three to five individual animals studied at each time point.

(~20-fold relative increase) in experimental mice grafted with C3H male hearts on day 6 posttransplant (n = 2–4 per group; Table I). Notably, PKH26-labeled Mar T cells that had not divided were detectable for >6 days in control animals not given transplants.

To more precisely determine the kinetics and location of indirect priming in vivo, we repeated the experiments at days 2 and 4 posttransplant. In mice that received male C3H allo grafts, the labeled Mar T cells were detectable in both the spleen and the lymph node on day 2 posttransplant, but the Mar cells had not yet divided (Fig. 3B, Table I). In contrast, essentially all of the Mar T cells divided by day 4, and the cell population markedly expanded in frequency by this time. Interestingly, the in vivo proliferation/activation occurred with equal rapidity and efficiency in the recipient spleen and the recipient peripheral lymph nodes, suggesting that T cell activation through the indirect pathway is a systemic event and is not localized to the spleen. The splenic Mar T cells in recipients of C3H male grafts (but not female grafts) down-regulated surface expression of CD62L and up-regulated expression of CD44 consistent with the development of an acutely activated phenotype (Fig. 4, Table I).

By gating on the CD4+ GFP− cell population, we could additionally determine the activation state of endogenous CD4+ T cells (Fig. 4). In contrast to the Mar T cells, only a portion of the endogenous host CD4+ T cells exhibited an activated CD44high CD62Llow phenotype, consistent with the fact that only a minority of the endogenous CD4+ T cells expressed alloreactive TCRs. The Mar T cells and host CD4+ T cells found in recipient peripheral lymph node expressed the same activated CD44high CD62Llow phenotype as those found in the spleen (data not shown).
Skin grafts are more immunogenic than heart grafts, so we additionally tested whether Mar T cells would respond to male B6 skin grafts in MHC class II-deficient hosts. As shown in Fig. 8, the transferred Mar T cells did not respond despite the fact that the MHC class II KO female grafts were rejected by naive male grafts (by day 10–12, n = 4; data not shown). In control experiments, Mar T cells did undergo activation and expansion in I-A\(^{b}\) WT B6 female recipients of B6 male skin (Fig. 8). Overall, the data strongly suggest that the indirect pathway is the dominant mode for presenting male Ag to recipient CD4\(^{+}\) T cells posttransplantation.

**In vivo trafficking patterns following indirect priming**

Previous studies of T cells specific for model Ags (24) and/or viral Ags (25) showed that priming and activation of T cells in vivo are associated with an ability to traffic broadly to peripheral organs. To determine whether CD4\(^{+}\) T cells indirectly primed by a cardiac transplant behave similarly, we next tested whether we could detect Mar T cells within peripheral organs. On day 5–6 posttransplant, lymphocytes infiltrating liver and lung tissue from animals adoptively transferred with Mar T cells and given male or female C3H heart grafts were isolated and evaluated by flow cytometry. Importantly, all of the organs were extensively perfused with PBS before isolation of the lymphocytes to minimize blood contamination of the samples. As shown in Fig. 9, activated Mar T cells (CD4\(^{+}\)CD62L\(^{low}\)) were readily detected in the liver (and the lung; not shown) of recipients of C3H male heart grafts. In contrast, essentially no Mar T cells were detectable in the liver of recipients transplanted with female hearts. Transplantation of both male and female C3H hearts resulted in activation of the alloreactive T cell repertoire, as CD62L\(^{low}\)CD44\(^{high}\)GFP CD4\(^{+}\) T cells were found in the peripheral organs of both sets of animals. The experiments confirm the hypothesis that indirectly primed CD4\(^{+}\) T cells traffic nonspecifically to peripheral organs.

Indirectly primed CD44\(^{high}\)CD62L\(^{low}\) Mar T cells were also readily detectable by flow cytometry within the GILs isolated from the male C3H heart grafts (Fig. 10A). No Mar T cells were detectable in the GILs isolated from female C3H hearts, corroborating the specificity of this result. To further validate the intragraft parenchymal infiltration of the indirectly primed Mar T cells, we performed two-color immunohistochemistry (Fig. 10B). Graft sections were stained with an anti-GFP Ab to identify the transferred Mar T cells (blue) and were costained with an Ab to recipient MHC II I-A\(^{b}\) (red/brown) to detect infiltrating recipient MHC class II-expressing APCs. The GFP transgenic Mar T cells were readily detectable in the male C3H heart grafts (Fig. 10B), substantiating the flow cytometry data of GILs (Fig. 10A) and consistent with trafficking of indirectly primed CD4 cells to the target organ in vivo. Essentially no GFP staining (representing Mar T cells) was detected in the specificity control, female C3H heart grafts. Quantitative image analysis was performed and revealed a statistically significant difference in the amount of anti-GFP (blue) staining detected in the male C3H hearts (>1200 ± 120 pixels per ×20 field, n = 3) vs the female C3H hearts (<120 ± 77 pixels per ×20 field, n = 3, p < 0.01; data not shown).

The anti-I-A\(^{b}\) Ab stained cells in both male and female C3H grafts (red color; Fig. 10). Immunohistochemistry using anti-F40/80 mAb showed a similar staining pattern (data not shown), consistent with the recipient I-A\(^{b}\) expression deriving from infiltrating macrophages/monocytes. Strikingly, we were able to detect the GFP\(^{+}\) Mar CD4\(^{+}\) T cells in close proximity to recipient MHC class II I-A\(^{b}\) APCs in the male, but not the female grafts (Fig. 10B, arrows). At least two to three of such blue/red couplets were found in every section of the C3H male hearts studied, while none were found in the sections of C3H female hearts (three individual animals were studied per group; data not shown). The reproducibility of the finding suggests an Ag-specific interaction between the two cell types within the graft itself. In sum, the results provide the first in vivo evidence that indirectly primed CD4 Mar T cells rapidly migrate to the target organ and strongly suggest that they re-encounter their specific ligand expressed on graft-infiltrating recipient APCs.

**Discussion**

Evidence derived from experiments performed over the last decade strongly supports the hypothesis that T cells responding through the indirect pathway participate in the graft rejection process. Despite this, a precise understanding of in vivo activation and migration of indirectly primed CD4\(^{+}\) T cells remained elusive, in part because of a lack of available models and reagents. The results presented in this study overcome this difficulty, and therefore offer new insight into in vivo mechanisms of transplant rejection. Our studies take advantage of TCR transgenic CD4\(^{+}\) T cells reactive to a donor-derived, immune dominant determinant that is naturally processed and presented by recipient APCs following heart transplantation (Fig. 1). The work illustrates several important issues that have not been previously addressed.

It is first notable that indirect priming of Mar CD4\(^{+}\) T cells occurred rapidly following transplantation. By day 4 after placement of the heart graft, well before significant damage occurred to the graft itself, essentially all of the recipient Mar T cells underwent >4 rounds of cell division and became CD62L\(^{low}\)CD44\(^{high}\)
There are a number of potential explanations to account for this result. Direct activation of naive donor-reactive CD4⁺ T cells may only occur during a time-limited window of opportunity, during which graft-derived dendritic cells migrate to recipient secondary lymphoid organs following transplantation and directly interact with recipient alloreactive T cells (26). In contrast, indirect priming can occur through multiple mechanisms and is, in theory, not time limited. Donor DCs are thought to undergo apoptosis in recipient secondary lymphoid organs, thereby triggering endocytosis by recipient DCs, and resulting in presentation of donor Ag in the context of recipient MHC class II (17, 27, 28). Priming through the indirect pathway could also occur when recipient macrophages enter the donor graft, endocytose donor Ag, process/present it in the context of recipient MHC (29), and then return to recipient secondary lymphoid organs to prime naive recipient T cells. This latter mechanism is unlikely to contribute to direct priming and could provide more opportunity for a specific T cell to encounter its alloantigen on a recipient APC as opposed to a donor APC. In addition, indirect presentation could theoretically occur when soluble, donor-derived molecules shed from the graft (30) drain through the bloodstream/lymphatics to the recipient secondary lymphoid organs where they would be processed and presented by recipient APCs. It seems unlikely that soluble Ag delivery to recipient APCs is the mechanism of Ag presentation in the present studies because the H-Y Ags are intracellular as opposed to membrane bound.

Perhaps more relevant to our studies is the fact that the processing and presentation of exogenous Ag preferentially lead to expression of peptides in the context of MHC class II molecules (31), while endogenous cytosolic proteins are preferentially expressed in the context of MHC class I (32, 33). It is possible that APCs from male B6 grafts do not express as many HYDhypo/L-Ab complexxes on their cell surface as recipient cells, because the H-Y Ag may be preferentially, although not exclusively, processed and presented in the context of MHC I on donor cells. In contrast, the endocytosis of donor male cells by recipient female B6 APCs post-transplant may preferentially lead to processing and presentation.

(Figs. 3 and 4), consistent with rapid activation. We found it particularly intriguing that Mar CD4⁺ T cells were efficiently primed through the indirect pathway, but the same monoclonal TCR transgenic T cells did not respond to donor male Ag in the absence of recipient MHC class II (Fig. 6, Table I). These unanticipated results show that, at least for HYDhypo-specific CD4⁺ T cells, the indirect pathway is more efficient at priming recipient T cells than the direct pathway.

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of HYDbyp-A^b complexes on recipient APCs at high levels. Unfortunately, there are no reagents available to specifically measure the amount of HYDbyp-A^b on the surface of a given cell. Nonetheless, the fact that Mar T cells in MHC class II-deficient hosts did not respond to male B6 heart nor skin grafts is consistent with the conclusion that insufficient quantities of HYDbyp/I-A^b were expressed on donor male APCs to lead to direct CD4^+ T cell activation. In further support of this contention, others have demonstrated that CD4^+ T cells can respond directly to other membrane-expressed donor alloantigens in the absence of recipient MHC class II (6, 7).

We found it notable that the indirect priming of Mar T cells occurred with similar kinetics in peripheral lymph nodes and spleen tissue, the latter of which is the site generally presumed to drain a heterotopic cardiac transplant. As heterotopic heart grafts do not have lymphatic drainage, it is reasonable to postulate that donor DCs may exit directly into the bloodstream, where they are circulated and distributed to a large number of lymphoid organs, where they subsequently and simultaneously activate naive CD4^+ T cells throughout the organism. The coincident activation in lymph node and spleen confirms functional studies performed by Lakkis et al. (2), showing that priming of naive alloreactive T cells in response to a cardiac transplant cannot occur in the absence of spleen and peripheral lymph node tissue, but can occur when either lymph node or spleen is present alone.

Finally, our data provide a first look at in vivo migratory patterns of indirectly primed CD4^+ T cells following heart transplantation. Activated Mar T cells in recipients of C3H male heart grafts were detected by flow cytometry in the liver on day 5 posttransplant. The migration to peripheral tissues was not an unusual feature unique to this TCR transgenic T cell, because activated recipient (endogenous) CD4^+ T cells were also detectable in the liver at the same time. Both the flow cytometry and immunohistochemistry studies further demonstrated that the indirectly primed CD4^+ T cells infiltrated the C3H male grafts by day 5–6 post-transplant. The immunohistochemistry results provide the first visual evidence that indirectly primed CD4^+ T cells can re-encounter their Ag on recipient APCs in the graft, a hypothesis that has long

**FIGURE 7.** Mar T cells parked in MHC class II KO mice are functional. Equal numbers of PKH26-labeled GFP^+ Mar T cells (4 × 10^6 per mouse) were adoptively transferred into WT B6 females (left) or MHC class II KO females (right). Three days later, the spleen cells were isolated and stimulated in vitro with supplementary WT B6 female APCs and HYDbyp (bottom) or OVA323–339 (top). The cultured cells were studied by three-color (CD4-PE, GFP, CD62L-PerCP) flow cytometry on day 7. CD62L histograms are gated on the GFP^+ cells. The resultant number of GFP^+ cells on day 7 was not statistically different between the HYDbyp-stimulated cultures isolated from WT vs the MHC class II KO mice and was 10- to 15-fold higher in the HYDbyp-stimulated cultures vs the OVA323–339-stimulated cultures. The experiment was repeated once with similar results.

**FIGURE 8.** Direct presentation of donor Ag by a skin graft does not efficiently prime Mar T cells. Enriched CD4^+ T cells from GFP^+ transgenic Mar RAG KO mice were injected into B6 female recipients of male (A) or female (B) C3H heart grafts. On day 5 posttransplantation, recipient liver tissue was perfused with PBS to remove endogenous blood and was digested with collagenase. The resultant cells were stained for CD4 (PE) and for activation markers CD44 or CD62L (PerCP). The transferred Mar T cells and the endogenous host CD4^+ T cells can be differentiated based on GFP expression. The results are fully representative of three individual animals studied in each group. Similar findings were noted using lung tissue from the same animals (data not shown).

**FIGURE 9.** Indirectly primed Mar T cells migrate to peripheral organs. CD4^+ T cells from GFP transgenic Mar RAG KO mice were injected into B6 female recipients of male (A) or female (B) C3H heart grafts. On day 5 posttransplantation, recipient liver tissue was perfused with PBS to remove endogenous blood and was digested with collagenase. The resultant cells were stained for CD4 (PE) and for activation markers CD44 or CD62L (PerCP). The transferred Mar T cells and the endogenous host CD4^+ T cells can be differentiated based on GFP expression. The results are fully representative of three individual animals studied in each group. Similar findings were noted using lung tissue from the same animals (data not shown).
Graft Infiltrating Lymphocytes

FIGURE 10. Indirectly primed Mar T cells migrate to male C3H heart grafts. A. CD4+ T cells from GFP transgenic Mar RAG KO mice were injected into B6 female recipients of male or female C3H heart grafts. On day 5 posttransplantation, GILs were isolated and stained for CD4 (PE) and for activation markers CD44 or CD62L (PerCP). The transferred Mar T cells and the endogenous host CD4+ T cells can be differentiated based on GFP expression. The results are fully representative of five individual animals studied in each group. B. Double-color immunohistochemistry staining of female (left, magnification ×20) or male (right, magnification ×40) heart graft sections using anti-GFP (blue) to detect Mar T cells and anti-I-A* (red/brown) to detect infiltrating recipient APCs. Multiple examples of apparent interactions between the Mar T cells and the recipient APCs (arrows) were detectable in each C3H male heart graft, but were not found in the C3H female hearts (see text for quantitative analysis). The images are fully representative of three individual animals studied per group.

References


