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J Immunol 2001; 166:4879-4883; doi: 10.4049/jimmunol.166.8.4879
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Acute Rejection in the Absence of Cognate Recognition of Allograft by T Cells

Michel Y. Braun, Isabelle Grandjean, Pascal Feunou, Livine Duban, Robert Kiss, and Olivier Lantz

We studied the effects of the indirect pathway of allograft recognition using T cells from TCR transgenic Marilyn mice, which recognize the male Ag H-Y in an I-A\textsuperscript{b}-restricted fashion. The T cells are not alloreactive to the H-2\textsuperscript{k} haplotype, because they are not activated when adoptively transferred into recombinase-activating gene-2\textsuperscript{−/−} common \(\gamma\)-chain\textsuperscript{−/−} double-mutant H-2\textsuperscript{k} male or female mice. However, skin from H-2\textsuperscript{k} males, but not from H-2\textsuperscript{k} females, is acutely rejected by recombinase-activating gene-2\textsuperscript{−/−} transgenic female recipients. In vitro, Marylin spleen cells primed by H-2\textsuperscript{k} skin grafting proliferated and secreted both IL-4 and IFN-\(\gamma\) in response to H-2\textsuperscript{k} male stimulators. However, the removal of H-2\textsuperscript{k} APC from the responding population abolished the response. Taken together, these results show that the indirect recognition that triggers rejection in this model is due to the recognition of H-Y Ag shed from H-2\textsuperscript{k} male allograft and presented by the recipient’s own I-A\textsuperscript{b} APC to transgenic T cells. This study demonstrates unequivocally the capacity of naive CD4\textsuperscript{+} T cells to promote the rejection of allografts through mechanisms that involve indirect destruction of grafted tissues. The Journal of Immunology, 2001, 166: 4879 – 4883.

More than 20 years ago, Lafferty and colleagues and, subsequently, Lechler and Batchelor demonstrated that the main immunogenic stimulus leading to graft rejection was provided by the migrant population of allogeneic “passenger” dendritic cells (DC) present in the allografted tissue (1–4). If the graft was depleted of these cells, rejection was less strong or was absent, and graft survival was extended. However, acute rejection was restored by injection of purified donor DC at the time of transplantation. To explain the variation in susceptibility to rejection observed in normal and passenger DC-depleted allografts, the authors put forward the hypothesis that there were two pathways for sensitization of allograft reactive CD4\textsuperscript{+} T cells in rejection responses. Route 1, the direct pathway of sensitization, involves the direct recognition of donor MHC molecules at the surface of donor stimulatory cells and requires the presence of donor-derived passenger DC in the graft (5). By route 2, the indirect pathway of sensitization, antigenic moieties derived from the graft are phagocytosed and processed by APC of recipient origin and presented as peptides in the binding groove of the recipient’s own MHC class II molecules. According to Lafferty’s hypothesis, the indirect pathway is the only route available for sensitization to donor passenger DC-depleted organs. Thus, direct cell-cell contact between alloreactive CD4\textsuperscript{+} T cells and graft cells would not be required to bring about rejection of passenger DC-depleted organs.

Ample evidence indicates that indirect allorecognition occurs during allograft rejection. The indirect pathway was put forward as a hypothesis to explain the Ag specificity of the CD4\textsuperscript{+} T cells responsible for the rejection of MHC class II Ag-deficient allografts (6). Several studies in humans, rats, and mice have revealed the presence of alloreactive CD4\textsuperscript{+} T cells specific to alloantigens presented as peptide fragments in association with recipient MHC molecules during allograft rejection (7–11). Moreover, it has been reported that intrathymic administration of allogeneic peptides that are known to stimulate self-restricted alloreactive T cell clones can prolong the survival of subsequent allografts, suggesting that indirect presentation is critical to the rejection process (12).

However, while it is clear that T cell clones sensitized to alloantigens through indirect recognition are present during the process of rejection, it has been hard to demonstrate whether they actually promote rejection. The main obstacle resides in the difficulty of finding situations where indirect sensitization represents the only pathway available for activation of alloreactive T cells. The most convincing approaches that have been tried to date involve either the transplantation of MHC class II molecule-deficient organs or the adoptive transfer of in vitro-derived T cells sensitized by indirect recognition of allopeptides (6, 13). Although elegantly designed, these experiments have major weaknesses. First, they fail to unequivocally show the complete absence of direct recognition of rejected grafts by adoptively transferred T cells. Moreover, they are not able to exclude the activity of unusual CD4\textsuperscript{+} T cells not restricted by conventional MHC class II molecules and capable of mediating allograft rejection (14–17). Finally, they do not demonstrate whether, in the absence of direct recognition, T cell priming by indirect recognition of allografts is sufficient to bring about rejection. This last point is of particular relevance because memory cells contribute a substantial proportion of the cells involved in primary responses stimulated by direct recognition of donor MHC molecules (18). In contrast, as for any

\*Laboratory of Experimental Immunology and †Department of Histopathology, Université Libre de Bruxelles, Brussels, Belgium; ‡Institut National de la Santé et de la Recherche Médicale, Unité 25, Hôpital Necker, Paris, France; and ¹Laboratoire d’Immunologie, Institut Curie, Paris, France

Received for publication October 18, 2000. Accepted for publication February 8, 2001.

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This work was supported by grants from the Fonds National de la Recherche Scientifique of Belgium, the Commission of the European Union (Grant BIO-CT97-2151), a Pôle d’Attraction Interuniversitaire of Belgium, the Association de la Recherche Contre le Cancer of France, the Agence Nationale de la Recherche sur le SIDA (France), the Fondation de la Recherche Médicale (France), the Institut National de la Santé et de la Recherche Médicale (France), and the Etablissement Français des Greffes (France).

Address correspondence and reprint requests to Dr. Michel Y. Braun, Laboratory of Experimental Immunology, Université Libre de Bruxelles, 808 route de Lennik, Brusells B-1070, Belgium. E-mail address: mbraun@ulb.ac.be

Abbreviations used in this paper: DC, dendritic cells; Tg, transgenic; RAG, recombinase-activating gene; \(\gamma\)-c, common \(\gamma\)-chain.
nominal protein Ag, peptides derived either from allogenic MHC molecules or from polymorphic proteins and presented in the context of the recipient’s own MHC molecules stimulate almost exclusively naive T cells.

To establish without ambiguity the capacity of naive CD4+ T cells to reject transplanted foreign tissue through the indirect recognition pathway, we analyzed the rejection of male H-2b skin allografts by recombination-activating gene-2 (RAG2)-deficient mice expressing an H-Y-specific I-A\textsuperscript{b}-restricted transgenic (Tg) TCR (Marilyn mice). We show here that in the strict absence of direct recognition, Marilyn CD4+ T cells were not only primed by, but were also able to acutely reject, a male H-2k skin allograft.

**Materials and Methods**

**Mice**

Female RAG2\(^{-/-}\) Marilyn mice, which are Tg for a TCR (V\(_{\alpha}1.1, V\beta8\)) specific for an H-Y peptide (NAGFNSNRANSSRSS) (19) presented by I-A\textsuperscript{b}, have been previously described (20) and were used as recipients of skin grafts. Male and female C3H (H-2\textsuperscript{b}) and C57BL/6 (B6) (H-2\textsuperscript{k}) mice were obtained from Harlan Netherlands (Horst, The Netherlands). Male and female H-2\textsuperscript{b} or H-2\textsuperscript{k}, RAG2\(^{-/-}\) common \(\gamma\)-chain (\(\gamma\)\textsuperscript{\text{c}}) double-deficient mice were obtained by crossing RAG2\(^{-/-}\) B6 (N9 to B6 from the CDTA, Orleans, France) with B10.BR and then with RAG2\(^{-/-}\) \(\gamma\)\textsuperscript{\text{c}} (N4 to B6 obtained from J. Di Santo, Paris, France) and were bred at the animal facility of Necker Hospital (Paris, France).

**Adoptive transfer and flow cytometry**

TCR Tg CD4+ T cells were purified using anti-CD4 magnetic beads and the VarioMacs system from Miltenyi Biotech (Paris, France). Highly purified (>95%) TCR Tg CD4+ T cells (1 \(\times\) 10\(^5\)) were injected i.v. into male and female RAG2\(^{-/-}\) \(\gamma\)\textsuperscript{\text{c}}-/- H-2\textsuperscript{b} recipients. Seven days later spleen cells were harvested, re-exclusively transferred animals, counted, and prepared for analysis by flow cytometry. APC-anti-CD4 (clone GK1.5), PE-anti-V\beta6 (clone RR4-7), biotin anti-CD26 (clone MEL-14), and FITC-anti-CD44 (clone IM7) mAbs were purchased from BD PharMingen (Meylan, France). Streptavidin TriColor was obtained from Caltag (Tebu, France).

Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Meylan, France).

**Skin graft and histology**

Female recipients were anesthetized and grafted on the left side of the back with tail skin (70 mm\(^2\)) from male donors. The grafts were secured using Vaseline gauze and a bandage. Bandages were removed on day 10, and the grafts were then visually scored daily for evidence of rejection. Grafts showing >50% necrosis were considered rejected. Skin grafts were collected from killed mice and stained with hematoxylin and eosin.

**Proliferation assay and determination of cytokine secretion**

Spleen cells (1 \(\times\) 10\(^5\)) isolated from TCR Tg animals at the time of skin graft rejection were mixed with titrated numbers of irradiated spleen cell stimulators in triplicates in U-bottom 96-well microplates. [\(^{3}\)H]Thymidine (1 \(\mu\)Ci) was added for the final 16 h of a 72-h incubation. In some experiments, culture supernatants were harvested and studied in a sandwich ELISA for cytokine production. ELISA kits for mouse IFN-\(\gamma\), IL-4, and IL-10 were purchased from BD Biosciences (San Jose, CA). The supernatants were added to wells coated with the respective antibodies, and incubated for 2 days at 37°C. Cytokine secretion was measured using commercially available diagnostic kits (Genzyme, Cambridge, MA). 

**Results and Discussion**

**Tg Marilyn CD4+ T cells do not directly recognize H-2\textsuperscript{k} alloantigens**

To determine whether CD4+ T cells reject allografts by the indirect pathway, we first tested the specificity of the Tg Marilyn CD4+ T cells. Because the Marilyn clone came from an H-2\textsuperscript{b}\textsuperscript{b}k F\(_1\) female, it seemed unlikely that it would react against H-2\textsuperscript{k}. However, reactivity against the higher dose of MHC molecules expressed by a homoyzous animal might be sufficient to activate the Tg T cells. Naive T cells from RAG2\(^{-/-}\) Tg Marilyn mice, which recognize the male Ag H-Y presented by I-A\textsuperscript{b} (20), were cultured with either irradiated H-2\textsuperscript{b} or H-2\textsuperscript{k} splenocytes. As shown in Fig. 1a, a vigorous proliferative response to H-2\textsuperscript{k} male stimulators was observed without any response toward H-2\textsuperscript{b}-expressing cells. From these results, one could conclude that Marilyn T cells do not directly recognize H-2\textsuperscript{k} allogeneic stimulators. However, because in vitro proliferation is not a sensitive method to test T cell reactivity, we also set up an in vivo model in which naive Marilyn T cells were adoptively transferred into H-2\textsuperscript{b} male hosts. To prevent T cell- or NK cell-mediated rejection of Marilyn cells, we used hosts deficient for the common cytokine \(\gamma\)-chain receptor and Rag (RAG2\(^{-/-}\) \(\gamma\)\textsuperscript{\text{c}}) (21, 22). Although these hosts express the H-Y protein, they do not express the I-A\textsuperscript{b} restriction element. Thus, their APC should not be capable of stimulating Marilyn T cells unless the H-Y-specific T cells can also cross-react against allochene H-2\textsuperscript{k} molecules. When injected into allochene RAG2\(^{-/-}\) \(\gamma\)\textsuperscript{\text{c}} male or female hosts (Fig. 1b), purified Marilyn T cells did not expand for at least 7 days and retained their naive phenotype as they continued to express low levels of CD44 and high levels of CD62L. This confirms that they were unable to recognize any Ag presented by H-2\textsuperscript{b} APC in the adoptive host. In contrast, they responded well to coinjected syngeneic male spleen cells, undergoing proliferation 7 days after transfer, with up-regulation of CD44 and down-regulation of CD62L (Fig. 1b). Thus, antigenic stimulation of Marilyn CD4+ T cells in the RAG2\(^{-/-}\) \(\gamma\)\textsuperscript{\text{c}}-/- H-2\textsuperscript{k} hosts was dependent on the cotransfer of H-Y-bearing I-A\textsuperscript{b} APC. Taken together, these results demonstrate that Marilyn CD4+ T cells do not directly recognize male or female cells expressing H-2\textsuperscript{k} alloantigens.
The activation status of the CD4<sup>+</sup> male hosts. Seven days later, the percentage and male hosts. The positive control condition in-
quantitative strengths of effector mechanisms of graft rejection
we did not notice any difference in the kinetics of the rejection
process.

Rejection of H-2<sup>k</sup> skin allograft does not polarize Marilyn
CD4<sup>+</sup> T cells toward Th1 or Th2 phenotype
We next analyzed the reactivity of Tg Marilyn T cells from mice
that had rejected H-2<sup>b</sup> male skin allografts. For MLR, spleen cells
were stimulated with irradiated H-2<sup>b</sup> male or female stim-
ulators. As depicted in Fig. 4, unpurified Marilyn spleen cells pro-
liferated in response to both H-2<sup>b</sup> and H-2<sup>b</sup> male cells, while they
did not respond to female stimulators. The cells also specifically
secreted both IFN-γ and IL-4 (Fig. 4). However, reactivity toward
lymphoid cells (Fig. 3). Taken together, these observations show
unequivocally that primary immunization of alloreactive CD4<sup>+</sup> T
cells by indirect recognition of graft Ags is sufficient by itself to
promote allograft rejection, therefore demonstrating indirect al-
lorecognition of grafted tissue as an efficient pathway for the re-
jection process.

We started these experiments in the hope of comparing the
quantitative strengths of effector mechanisms of graft rejection me-
diated by direct vs indirect alloantigen presentation and to analyze
the immunohistopathology characterizing each system. However,
we did not notice any difference in the kinetics of the rejection
responses for H-2<sup>b</sup> male skin (indirect) and H-2<sup>b</sup> male skin (direct
and indirect; data not shown). Moreover, careful analysis of the
histopathology associated with the two types of rejection did not
reveal differences (data not shown). Thus, it would appear that the
effector mechanisms used by T cells sensitized by direct or indirect
allorecognition in our Tg model do not promote distinct pathology
in rejected skin.

The striking features of the rejection of an allograft are the spe-
cific destruction of graft elements and the absence of significant
damages to host tissues that are close to or in direct contact with
the graft. These have led to the widely accepted view that immu-
nologically specific mechanisms of tissue destruction, i.e., CTL,
activity, Abs, etc., are responsible for the rejection of allografts.
Early works on the rejection of mosaic skin from allophenic mice
grafted onto one of the parent strains provide direct evidence for
the Ag specificity of the effector mechanism of allograft rejection
(24–26). In these grafts only cells that express the target allo-
tigen are destroyed, whereas cells that are syngeneic to the recip-
ient survive indefinitely. The absence of substantial damage to
hystander syngeneic cells in the graft has fostered the idea that
allograft rejection is the result of specific killing of graft cells by
humoral or cell-mediated cytotoxicity. However, several observa-
tions suggest that nonspecific mechanisms of tissue destruction
also participate in graft rejection. First is the observation that the
syngeneic elements of allophraphic grafts endure some degree of
damage during rejection on parental recipients, but unlike the
allogenic compartment of the grafts, they are not fully destroyed,
and most of them survive indefinitely (25). A second piece of
evidence is the rejection of chimeric skin in which graft paren-
chyma cells are syngeneic to the recipient and only skin passenger
leukocytes, i.e., Langerhans cells, express allogenic MHC mole-
cules (27). In this setting acute rejection would be the direct result
of immunologically nonspecific destruction of graft cells. Our
study demonstrates the capacity of immunologically nonspecific
effector mechanisms to bring about graft rejection. Because of
their genetic deficiency, RAG2<sup>−/−</sup> TCR Tg recipients do not con-
tain mature B or T cells other than those expressing the TCR
transgene and thus cannot develop Ag-specific effector mecha-
nisms of rejection against male H-2<sup>b</sup> allografts.

Rejection of an allograft does not polarize Marilyn
CD4<sup>+</sup> T cells toward Th1 or Th2 phenotype
We next analyzed the reactivity of Tg Marilyn T cells from mice
that had rejected H-2<sup>b</sup> male skin allografts. For MLR, spleen cells
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liferated in response to both H-2<sup>b</sup> and H-2<sup>b</sup> male cells, while they
did not respond to female stimulators. The cells also specifically
secreted both IFN-γ and IL-4 (Fig. 4). However, reactivity toward
H-2k male cells was completely abrogated by depletion of MHC class II Ag-expressing cells from the responding population (Fig. 4). This observation confirms that Marilyn’s reactivity toward H-2k male cells is indeed the result of indirect recognition of H-Y presented by I-Ab and requires H-2b APC in the assay. The fact that IFN-γ and IL-4 were secreted in vitro by Marilyn T cells after stimulation with male cells suggests that both Th1- and Th2-dependent effector pathways of rejection operate in this system. Among pathways considered as being Th1 mediated, classical CTL can be ruled out, because it requires cognate recognition between effector CD4+ T cells and target graft cells. However, because keratinocytes, like hepatocytes, are sensitive to CD95-mediated apoptosis and express CD95L following exposure to inflammatory cytokines (28), one should not discard a possible role for Fas-mediated apoptosis of keratinocytes in the rejection process (29). The presence of numerous macrophage-like cells in allograft infiltrates suggests a delayed-type hypersensitivity-like reaction to be the main pathway of rejection. In contrast, the presence of eosinophilic infiltrates in rejected allografts supports the idea that a Th2-dependent pathway of rejection involving IL-5 and eosinophils may be operating in our model (30). Because we used RAG2−/− recipients, a role for B cell Abs and classical CTL in the rejection process can be ruled out.

**Concluding remarks**

It is often assumed that, during rejection, the peptides recognized by indirect pathway CD4+ T cells come mainly from the processing and presentation by recipient APCs of polymorphic moieties derived from donor MHC molecules (31). Our study confirms early findings by Wettstein et al. (32) that rejection by T cells also involve the indirect recognition of peptides from minor transplantation Ags. Thus, minor histocompatibility Ags, such as H-Y Ag and polymorphic molecules, must also be considered as a source of alloepitopes for indirect allorecognition in the design of peptide-based immune intervention that would interfere with the rejection process.

The important finding of our study is that the indirect pathway of alloantigen recognition can alone mediate the acute rejection of fully histoincompatible allografts. Because we are using TCR Tg recipients, one could question the relevance of such an observation. Indeed, it could be argued that this situation results from the stimulation of an abnormally high number of indirect pathway T cells and that this would not occur in non-Tg recipients. In animal models, direct pathway T cells have been estimated to represent >90% of the T cell repertoire participating in the process of acute rejection, whereas indirect pathway T cells would include only 1–10% (11). However, one should not forget that the ratio of direct-over-indirect pathway T cells might be profoundly perturbed in clinical situations where sensitization to donor alloantigens has taken place before transplantation (33, 34). Indeed, previous exposure to antigenic peptides has been reported to induce dramatic expansion of Ag-specific oligoclonal T cell populations (35–37). Thus, in some donor/recipient combinations, indirect pathway T cells may dominate the alloresponse and mediate allograft rejection.

### Responders:

**Whole Spleen Cells**

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**Purified T Cells**

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<td>H-2k females</td>
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**FIGURE 3.** Histology of allogeneic RAG2−/− H-2k male skin grafts rejected by I-Ab-restricted H-Y-specific TCR Tg Marilyn mice. Female and male skin allografts were placed onto Marilyn recipients. a, No rejection at day 30 after placement of female graft. b, Rejection on day 11 of male graft. Note extensive infiltration by lymphoid cells and absence of hair follicles. Lymphocytes (c), macrophage-like cells (d; blue arrows), and eosinophils (d; red arrows) were observed in rejected allografts. Hematoxylin and eosin stain; magnification, ×200 (a and b) and ×1000 (c and d).

**FIGURE 4.** In vitro activation of Marilyn H-Y-specific TCR Tg T cells in response to H-2k male stimulators depends on the presence of I-Ab-expressing APC in the assay. Whole spleen cells (a–c) or purified splenic T cells (d–f) from Marilyn TCR Tg mice that had rejected male H-2k skin graft were stimulated for 72 h in mixed lymphocyte cultures with irradiated male (○ and △) or female (● and ▲) H-2k C57BL/6 (○ and △) or H-2b C3H (● and ▲) stimulators. The proliferative response was measured during the last 16 h of culture (a and d).Culture supernatants were also harvested after 72 h, and levels of IFN-γ (b and e) and IL-4 (c and f) were determined by sandwich ELISA. Results are representative of two independent experiments.
Acknowledgments

We thank P. Matzinger for her helpful comments on the manuscript.

References


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