Cutting Edge: Alloimmune Responses Against Major and Minor Histocompatibility Antigens: Distinct Division Kinetics and Requirement for CD28 Costimulation

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Comparative study of alloimmune responses against major and minor histocompatibility Ags has been limited by the lack of suitable assays. Here, we use a bioassay that permits tracking of alloreactive CD4+ T cells populations as they proliferate in response to major or minor histocompatibility Ags in vivo. Division of alloreactive CD4+ T cells proceeded more rapidly in response to major histocompatibility Ags than minor Ags, although CD4+ T cells alloreactive to major Ags had a similar capacity to divide successively up to eight times after stimulation. Allore cognition of minor histocompatibility Ags was highly dependent on CD28 costimulation, with the frequency of CD4+ T cells proliferating in response to major Ags in the absence of CD28 costimulation reduced up to 20-fold. These findings highlight differences in signaling processes that lead to allore cognition of major and minor histocompatibility Ags and have implications on the design of interventions aimed at abrogating these responses. The Journal of Immunology, 1999, 162: 2467–2471.

Alloimmunity represents a markedly polyclonal response of the immune system against histocompatibility Ags expressed by transplanted tissues. CD4+ T cells are central to this response, being absolutely required to initiate rejection of allografts in certain settings (1–3). CD4+ T cells recognize major and minor histocompatibility Ags via distinct pathways. CD4+ T cells recognize MHC class II molecules of donor origin with bound peptide ligands on the surface of allogeneic cells (4–6).

Minor histocompatibility Ags are peptides recognized by CD4+ T cells in the context of donor MHC class II when the donor and recipient are MHC identical and in the context of self-MHC class II after processing by recipient APCs (7, 8).

Promiscuous recognition of allogeneic MHC with peptide ligands is thought to provoke potent T cell responses against major histocompatibility Ags, represented in vitro by vigorous primary MLR (6, 9–11). On the other hand, the study of alloimmune responses against minor histocompatibility Ags has been limited by the unavailability of assays that can detect the response of a T cell repertoire to minor Ags. Minor histocompatibility Ags fail to induce detectable T cell proliferation in vitro despite the fact that transplanted tissues mismatched for minor Ags are rejected almost as rapidly as MHC incompatible allografts (12). Therefore, comparison of the cumulative response of a T cell population against major and multiminor histocompatibility Ags has not been feasible. In this study, we use a bioassay that permits characterization of the in vivo kinetics of CD4+ T cell division in response to major as well as minor histocompatibility Ags. In addition, we demonstrate distinct requirements for CD28 costimulation in CD4+ T cell responses against major and minor histocompatibility Ags.

Materials and Methods
Mice
C57BL/6 (H-2b), C57BL/6-Cd28−/− (H-2b), BALB/c (H-2d), C3H/HeJ (H-2h), and C3H.SW-H2d/SnJ (H-2d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6TacBr−/−[KO]A−/− mice were purchased from Taconic (Germantown, NY). C57BL/6-Cd28−/− and C57BL/6TacBr−/−[KO]A−/− mice are homozygous for targeted disruption of the CD28 gene (CD28−/−) and Aβ (H-2d) gene (MHC class II−/−), respectively (13, 14). C3H.SW-H2d/SnJ mice are congenic for the H-2d allele (15).

In vivo MLR
Wild-type and CD28−/− C57BL/6 mice were designated as responder strains and C3H/HeJ and C3H.SW mice as stimulator strains. In addition, we wished to use stimulator mice that were deficient in MHC class II. Because of the unavailability of MHC class II−/− C3H mice, mice class II−/− C57BL/6 mice were used as stimulators with BALB/c responders. Lymphocytes were isolated from peripheral lymph nodes and spleens of 8- to 10-wk-old responder strain mice.
mice, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) \(^3\) (Molecular Probes, Eugene, OR) as previously described, and transferred via tail vein into whole-body irradiated (1800 rad) stimulator mice (30–40 million lymphocytes/stimulator) \(^1\). Responder cells were recovered from stimulator spleens to assure sufficient numbers of lymphocytes for flow cytometric analysis at serial time points.

**Flow cytometry**

Harvested lymphocytes were stained with phycoerythrin-conjugated anti-CD4 mAb (GK1.5; PharMingen, San Diego, CA) and analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). CD4\(^+\) T cells of responder strain origin were identified in the CFSE\(^1\)CD4\(^+\) gate.

**Calculation of alloreactive precursor frequencies**

Precursor frequencies of alloreactive CD4\(^+\) T cells were calculated as previously described \(^1\). Briefly, discrete generations of alloreactive CD4\(^+\) T cells were identified by their CFSE fluorescence. The number of alloreactive CD4\(^+\) T cells in each daughter generation was counted using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). CD4\(^+\) T cells of responder strain origin were identified in the CFSE\(^1\)CD4\(^+\) gate.

\[ \frac{a}{2^n} \]

where \(a\) is the number of cells in generation, \(n\) is the generation. For example, 8 daughter cells counted in the third daughter generation would be extrapolated to be the progeny of 1 precursor that divided three times \((8/2^3 = 1)\). The frequency of alloreactive CD4\(^+\) T cells in the responder population was then calculated by dividing the total number of precursors from all daughter cells that proliferated beyond background levels by the sum of all precursors and undivided cells.

\(^3\) Abbreviation used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester.

**Results and Discussion**

**Kinetics of CD4\(^+\) T cell division in response to major and minor histocompatibility Ags**

Major histocompatibility Ags are known to yield vigorous primary MLR; however, these assays typically measure incorporation of tritiated thymidine, which only gives a measure of DNA synthesis in vitro \(^1\). To characterize the kinetics of alloreactive CD4\(^+\) T cell division in vivo, we used the fluorescein-based dye CFSE. Labeling of lymphocytes with CFSE yields a homogenously stained population that fluoresces at wavelengths characteristic of the fluorescein moiety of CFSE \(^1\). CFSE-labeled C57BL/6 (H-2\(^b\)) responder lymphocytes were transferred into whole-body irradiated C57BL/6 (A), C3H/HeJ (B), and C3H.SW (C) stimulator mice. Responder lymphocytes were retrieved from the spleens of stimulator mice and analyzed for CFSE fluorescence by flow cytometry. In Fig. 1, histograms depict CFSE fluorescence of CFSE\(^1\)CD4\(^+\) cells. Undivided parent generation CD4\(^+\) T cells are represented by the peak of highest CFSE fluorescence in each histogram, and alloreactive daughter generations are represented by leftward peaks of diminishing CFSE fluorescence. Results are representative of at least 10 independent experiments.

**FIGURE 1.** In vivo kinetics of CD4\(^+\) T cell division after transfer into syngeneic, major histocompatibility Ag-mismatched, and minor Ag-mismatched mice. CFSE-labeled C57BL/6 responder lymphocytes were transferred into whole-body irradiated C57BL/6 (A), C3H/HeJ (B), and C3H.SW (C) stimulator mice and harvested from spleens of stimulators at serial time points. Histograms depict CFSE fluorescence of CFSE\(^1\)CD4\(^+\) cells. Undivided parent generation CD4\(^+\) T cells are represented by the peak of highest CFSE fluorescence in each histogram, and alloreactive daughter generations are represented by leftward peaks of diminishing CFSE fluorescence. Results are representative of at least 10 independent experiments.
this assay. In contrast, responder CD4\(^+\) T cells proliferated vigorously after transfer into MHC-mismatched C3H/HeJ stimulators (Fig. 1B). The onset of proliferation was rapid, with alloreactive cells dividing to the fourth daughter generation after 48 h. Proliferation continued through subsequent time points such that by 72 h large populations of seventh and eighth generation alloreactive progeny were recovered. The step-wise production at serial time points of successively dividing daughter generations suggests that proliferating cells represent the progeny of alloreactive cells that underwent in situ activation and division.

Compared with the response against major histocompatibility Ags, the CD4\(^+\) T cell response against minor Ags encoded by C3H.SW stimulators was delayed and the frequency of alloreactive cells was decreased (Fig. 1C). Division to the third daughter generation by a subset of alloreactive cells was detected after 48 h. At subsequent time points, alloreactive cell division continued to trail the response to major Ags such that seventh and eighth generation progeny accumulated in stimulator spleens only after 84 h. The lower frequency of CD4\(^+\) T cells dividing after transfer into minor histocompatibility-mismatched stimulators was apparent because of the small size of daughter generation peaks relative to the undivided parent generation peak.

**Direct recognition of allogeic MHC class II by responder cells**

We hypothesized that the proliferative responses of alloreactive responder cells resulted from direct recognition of allogeic MHC class II on the surface of APCs of stimulator origin. To evaluate this, we used stimulator mice deficient in MHC class II. In contrast to the vigorous proliferation by alloreactive CD4\(^+\) T cells after transfer into wild-type stimulators, no proliferation was detected 60 h after transfer into MHC class II\(^-/-\) stimulators (Fig. 2). This finding confirms that in this experimental system alloreactive CD4\(^+\) T cell proliferation is dependent upon direct recognition of allogeic MHC class II. Indirect recognition of histocompatibility Ags processed and presented in the context of self-MHC class II by APCs of responder strain origin does not contribute significantly to the observed proliferative responses. The practical implication of this finding is that direct allorecognition per se can be studied in this system without the potentially confounding presence of the indirect pathway.

**Allorecognition of major and minor histocompatibility Ags by CD28\(^-/-\) responder cells**

To assess the role of CD28 costimulation in allorecognition of major and minor histocompatibility Ags, we transferred CFSE-labeled responder cells from CD28\(^-/-\) C57BL/6 mice into C3H/HeJ and C3H.SW stimulators. CD28\(^-/-\) responder CD4\(^+\) T cells divided less rapidly than wild-type responder cells in response to major histocompatibility Ags. Although a subset of alloreactive CD28\(^-/-\) CD4\(^+\) T cells divided to the sixth daughter generation after 60 h (Fig. 3A), the proportion of alloreactive cells dividing maximally at this time point was lower than that of wild-type responder cells (Fig. 3B). Despite this slowing in division kinetics, alloreactive CD28\(^-/-\) CD4\(^+\) T cells did retain the capacity to divide successively such that the majority of alloreactive progeny accumulated in the seventh and eighth daughter generations after 84 h (data not shown). In contrast, proliferation of responder CD4\(^+\) T cells in response to minor Ags demonstrated marked dependence on CD28 costimulation. Minimal division by CD28\(^-/-\) CD4\(^+\) T cells was detected 84 h after transfer into minor histocompatibility-mismatched stimulators (Fig. 4A), in contrast to division to seventh and eighth generations by wild-type CD4\(^+\) T cells (Fig. 4B).

To further define the role of CD28 costimulation in the allorecognition of histocompatibility Ags, we calculated precursor frequencies of alloreactive wild-type and CD28\(^-/-\) CD4\(^+\) T cells that were present in harvested spleens of stimulators over time. The frequencies calculated for CD4\(^+\) T cells dividing in response to C3H/HeJ stimulators were high, peaking at 21 ± 2\% 60 h after transfer before declining to 15 ± 2\% and 14 ± 2\% at subsequent time points (Fig. 5A). The decreasing calculated frequencies at
delayed kinetics of alloreactive cell division in response to minor increasing pattern of calculated frequencies is consistent with the compatibility Ags, while a much larger proportion of CD4 cells requires CD28 costimulation for recognition of major histocompatibility Ags (6, 21, 22). We attribute this discrepancy to sequestration and enrichment of alloreactive to minor Ags requires CD28 costimulation rather than self-MHC led to more rapid division by alloreactive CD4 T cells responding to minor histocompatibility Ags.

The precursor frequency values calculated by these means are uniformly higher than previous estimates of 1–10% for CD4 T cells alloreactive to major histocompatibility Ags in the context of self-MHC may be more susceptible to such processes that lead to recognition of major histocompatibility Ags were modestly decreased when compared with wild-type responder cells (Fig. 5A). In contrast, precursor frequencies of CD28−/− CD4+ T cells alloreactive to minor histocompatibility Ags were sharply decreased at all time points compared with wild-type responder cells (Fig. 5B; 0.24 ± 0.05% vs 5.6 ± 0.8%, respectively, after 84 h; p < 0.01). Collectively, these findings indicate that a small subset of alloreactive CD4 T cells alloreactive to major histocompatibility Ags, while a much larger proportion of CD4+ T cells potentially alloreactive to minor Ags requires CD28 costimulation for allorecognition to occur.

The precursor frequency values calculated by these means are uniformly higher than previous estimates of 1–10% for CD4+ T cells alloreactive to major histocompatibility Ags (6, 21, 22). We attribute this discrepancy to sequestration and enrichment of alloreactive T cells. Results are representative of at least five independent experiments.

FIGURE 4. Dependence of CD4+ T cells on CD28 costimulation for allorecognition of minor histocompatibility Ags. CFSE-labeled responder lymphocytes from CD28−/− (A) and wild-type (B) C57BL/6 mice were transferred into whole-body irradiated C3H.SW stimulator mice and harvested from spleens of stimulators after 84 h for flow cytometric analysis. Proliferation of CD28−/− CD4+ T cells in response to minor histocompatibility Ags was greatly reduced compared with wild-type responder cells. Results are representative of at least five independent experiments.

FIGURE 5. Mean precursor frequencies of wild-type and CD28−/− CD4+ T cells alloreactive to major and minor histocompatibility Ags. Precursor frequencies of alloreactive wild-type (WT) and CD28−/− C57BL/6 CD4+ T cells were calculated after harvesting responders at serial time points after transfer into C3H/HeJ (A) and C3H.SW (B) stimulator mice. The frequency of CD4+ T cells alloreactive to major histocompatibility Ags was marginally decreased in the absence of CD28 costimulation while the frequency of CD4+ T cells responding to minor histocompatibility Ags was sharply decreased. Error bars represent SD of at least four experiments for each time point.
interventions. Therefore, prolongation of allograft survival by CD28 costimulatory blockade suggests that indirect presentation of histocompatibility Ags may be the dominant pathway of Ag presentation leading to rejection in these transplantation models. Future studies using this experimental system may define how other signaling pathways help set the threshold for allorecognition and allow efficient testing of strategies aimed at abrogating these responses.

References


