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*J Immunol* 2012; 188:2703-2711; Prepublished online 10 February 2012; doi: 10.4049/jimmunol.1102997

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Innate NK Cells and Macrophages Recognize and Reject Allogeneic Nonself In Vivo via Different Mechanisms

Wentao Liu,* Xiang Xiao,* Gulcin Demirci,* Joren Madsen,† and Xian C. Li*

Both innate and adaptive immune cells are involved in the allograft response. But how the innate immune cells respond to allotransplants remains poorly defined. In the current study, we examined the roles of NK cells and macrophages in recognizing and rejecting allogeneic cells in vivo. We found that in naive mice NK cells are the primary effector cells in the killing of allogeneic cells via “missing self” recognition. However, in alloantigen-presensitized mice, NK cells are dispensable. Instead, macrophages become alloreactive and readily recognize and reject allogeneic nonself. This effect requires help from activated CD4+ T cells and involves CD40/CD40L engagement, because blocking CD40/CD40L interactions prevents macrophage-mediated rejection of allogeneic cells. Conversely, actively stimulating CD40 triggers macrophage-mediated rejection in the absence of CD4+ T cells. Importantly, alloantigen-primed and CD4+ T cell-helped macrophages (licensed macrophages) exhibit potent regulatory function in vivo in an acute graft-versus-host disease model. Together, our data uncover an important role for macrophages in the alloimmune response and may have important clinical implications. The Journal of Immunology, 2012, 188: 2703–2711.

With few exceptions, a traditional view in transplantation (both bone marrow and solid organs) is that T cells are necessary and sufficient for allograft rejection. This view comes from the observation that rejection often does not occur in naive hosts in the absence of T cells (1). However, the destruction of an allograft in the effector phase of a rejection response is far more complex, involving many other cell types besides T cells. In fact, innate immune cells, such as NK cells and macrophages, are often well represented in the rejecting allografts (2, 3). As compared with T cells, much less is known about how innate immune cells influence the allograft response (graft rejection or acceptance). The common wisdom is that innate cells do not respond directly to allotransplants, but they can be brought in by activated T cells to aid the process of graft destruction. Thus, innate immune cells are thought to play a redundant or even a dispensable role in the rejection response (4).

There are several lines of evidence now that call for a reassessment of the role of innate immune cells in transplant models. For example, a seminal work by Murphy et al. (5) in 1987 revealed that NK cells can mediate the specificity of bone marrow graft rejection. We now know that NK cells are equipped with a unique receptor system that allows them to recognize and reject allogeneic cells via “missing self” or “missing ligand” recognition (6), and because of this, NK cells are in fact alloreactive and capable of rejecting allotransplants (both bone marrow cells and solid grafts) (7–10).

Some of the cell surface molecules on NK cells (e.g., NKG2D) have been identified as key activators of NK cells in the rejection of bone marrow grafts (11). Interestingly, NK cells also can acquire additional features that are ascribed traditionally to adaptive T cells and B cells; NK cells can respond in an Ag-specific manner, undergo clonal expansion before becoming effector cells, and even acquire memory features capable of mediating recall responses (12). These unexpected findings drastically expand the role of NK cells in overall immune responses. Besides NK cells, macrophages, which are thought to be nonspecific inflammatory cells, also can exhibit a certain degree of specificity in selected transplant models. To this end, Zecher et al. (13) recently reported that the challenge of Rag2<sup>−/−</sup> mice with alloantigens sensitizes host monocytes/macrophages that subsequently mediate an alloantigen-specific delayed-type hypersensitivity response, suggesting that monocytes/macrophages may express a previously unknown allore cognition system that allows self/nonself discrimination. In fact, in a xenogeneic islet transplant model (porcine to mouse), macrophages turn out to be the primary effector cells in the destruction of islet transplants (14, 15). After being sensitized and activated by porcine xenothermalts, macrophages exhibit a high degree of Ag specificity in that they selectively and specifically destroy porcine islet transplants to which they are sensitized but not allogeneic islets (14, 15). Similar findings also were reported in other settings (16). Considering the prevailing view of T cells in transplant models, these findings may be viewed simply as exceptions. However, these data do raise significant questions regarding the exact role of innate immune cells in response to allotransplants. The recent demonstration that kidney allograft rejection in humans after aggressive T cell depletion therapies is dominated by innate immune cells, especially monocytes/macrophages, suggests that these cells may play a much larger role in transplant rejection than that envisioned previously (17).

In the current study, we took an in vivo approach to examine innate immune responses to allogeneic nonself cells immediately after alloantigen encounter (within the first 16 h) or weeks thereafter. We focused on NK cells and macrophages in this setting and found striking differences in the alloreactivity of NK cells and macrophages in naive versus alloantigen-presensitized hosts. Specifically, NK cells reject allogeneic nonself cells in naive mice, but they are dispensable in donor Ag-presensitized hosts.
expectedly, macrophages can be driven to an alloreactive mode in alloantigen-presented hosts and mediate the rejection of allogeneic cells. This effect requires help from activated CD4⁺ T cells and involves CD40/CD40L interactions. Importantly, alloreactive macrophages display potent regulatory functions in vivo in an acute graft-versus-host disease (GVHD) model.

Materials and Methods

Animals

Wild-type C57BL/6 mice (H-2b), perforin knockout, CD4 knockout (CD4KO), CD8 knockout (CD8KO), CD11b-GFP-DTR transgenic, and Rag⁻/⁻ mice on the C57BL/6 background (H-2b) were purchased from The Jackson Laboratory (Bar Harbor, ME). DBA/2 and BALB/c mice (H-2b) also were obtained from The Jackson Laboratory. Rag⁻/⁻ γc⁻/⁻ double-mutant mice (H-2b) and C5H (H-2b) mice were obtained from Taconic Farms (Germantown, NY). Animal care and use conformed to the guidelines established by the Animal Care Committee at Harvard Medical School in Boston, MA.

Agbs and reagents

The following anti-mouse mAbs used for cell surface staining were obtained from eBiosciences (San Diego, CA): PE-Cy7-anti-CD4 (clone GK1.5), Pacific blue-anti-CD11b (clone M1/70), PE-Cy5-anti-F4/80 (clone BM8), FITC-anti-αβ (clone FAL11), PE-anti-CD40 (clone IC01), PE-anti-CD80 (clone M89-61), allopoxycyanin-anti-CD11c (clone N418), PE-Cy7-anti-NK2.2 (clone C25), PE-Cy7-anti-CD62L (clone MEL-14), Pacific blue-anti-CD26 (clone MEL-14), PE-anti-CD44 (clone IM-7), and isotype control Abs. PE-anti-Ly-6c (clone HK14) was purchased from Southern Biotech (Birmingham, AL). Anti-CD40L (clone MR1), anti-NK1.1 (clone PK136), and anti-CD4 mAb (clone GK1.5) were purchased from BioXcell (West Lebanon, NH) and used for in vivo experiments. An agonist anti-CD40 (clone FGGK45.5) mAb was purchased from Millenyi Biotec (Bergisch Gladbach, Germany) and used for some in vivo experiments.

In vivo killing assay

This assay was performed as described previously (9). Briefly, spleen cells were prepared from allogeneic donors, namely, BALB/c and C3H/He mice, and syngeneic C57BL/6 mice, and the cells were labeled with 0.2 and 2.5 μM CFSE ex vivo, respectively. The CFSE-labeled allogeneic and syngeneic cells were mixed together at 1:1 ratio, and the cell mixture was injected into host mice via the tail vein. Each mouse received ∼10⁶ cells. The host mice were killed 16–17 h later, and the survival of CFSE-labeled donor cells in the host spleen was analyzed using a FACSscan flow cytometer by selectively gating onto the CFSE-positive cells. The ratio of CFSE-labeled cells was determined by FACS and is shown as a relative percentage in histograms. The rejection of donor cells was calculated using the formula [1 – CFSE⁺ events/CFSE⁺ events] × 100% and then compared among different groups.

Cell staining and flow cytometry

Spleen and lymph node cells were harvested, a single-cell suspension was prepared, and cells were resuspended in PBS/0.5% BSA and stained with fluorochrome-conjugated Abs on ice for 30 min. The cells were washed twice in PBS/BSA and fixed in 1% paraformaldehyde before FACS analysis. All of the samples were acquired using the FACScan or LSRII (BD Biosciences, San Jose, CA), and the data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Quantitative real-time PCR

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA with the ABI PRISM TaqMan reverse transcription method (9). The expression of the genes of interest and of the GAPDH control was assessed in simplex RT-PCR with fluorescent probe for detecting NO-producing cells (Invitrogen, Carlsbad, CA), at 37°C for 40 min in an incubator with 5% CO₂. After the incubation, cells were washed with fresh RPMI 1640 medium and further incubated for an additional 20 min. After that, cells were incubated with an Fc blocker, followed by staining with anti-CD11b and anti-F4/80 Abs. NO production by macrophages was assessed by the intensity of green fluorescence (excitation/emission at ∼490/515 nm) in the F4/80⁺ population, and data were presented in histograms.

Reactive oxygen species production

Reactive oxygen species (ROS) production was assessed with a fluorogenic assay (19). Briefly, macrophages (2 × 10⁶ cells/200 μl) were incubated with 1 μM aminophenyl fluorescein, a green fluorescent probe for detecting ROS (Invitrogen), at 37°C for 40 min in an incubator with 5% CO₂. After the incubation, cells were washed to remove excessive probe, followed by blocking with an Fc-blocker. Cells were stained further with anti-CD11b and anti-F4/80 Abs. ROS production by macrophages was assessed by FACS by assessing the intensity of green fluorescence (excitation/emission at ∼490/515 nm) in the F4/80⁺ population.

Isolation of macrophages

Mice were anesthetized, a ventral midline incision exposed the peritoneal cavity, and a sterile 24-gauge cannula was inserted into the vena porta. The liver was perfused in situ with collagenase A solution (0.05% in RPMI 1640) at a flow rate of 6 ml/min for 30 s at 35°C. After the perfusion, the liver was excised, sliced, and further incubated for 20 min, followed by gently pushing the digested tissue through a nylon filter (88 μm). The cell suspension was collected, and the cells were pelleted by centrifugation and then washed twice at 500 × g for 8 min. Cells were resuspended in PBS and gently layered on a double Percoll gradient (30% and 70%), followed by centrifugation at 800 × g for 15 min. Cells at the 30–70% gradient interface were collected, and macrophages (CD11b⁺F4/80⁺) were sorted further using a MoFlo high-speed cell sorter.

Cell depletion in vivo

Depletion of NK cells in vivo was accomplished using a depleting anti-NK1.1 mAb (clone PK136); the mAb was given at 0.25 mg i.p. for 2 consecutive days. CD4⁺ T cells were depleted with a depleting anti-CD4 mAb (clone GK1.5); mice were injected with the anti-CD4 mAb at 0.25 mg i.p. for 2 consecutive days. Cell depletion with these protocols has been always >90%, as assessed by FACS (20). Gadolinium chloride (Sigma-Aldrich, St. Louis, MO) was used to inhibit macrophages in vivo (21). Mice were given gadolinium chloride at 20 mg/kg/day for 3 consecutive days before performing the in vivo killing assays.

Adaptive cell transfer

In some experiments where immunodeficient Rag⁻/⁻ or Rag⁻/⁻γc⁻/⁻ mice were used as hosts, CD4⁺ T cells from C57BL/6 mice were prepared by MACS, and 20 × 10⁶ cells were injected into each host via the tail vein.

Acute GVHD in vivo

Male Rag⁻/⁻γc⁻/⁻ mice (H-2b) were used as hosts, and each host was given 40 × 10⁶ allogeneic BALB/c T cells via the tail vein to induce the graft-versus-host response. The severity of the response was assessed by weight loss and tissue pathology. Groups of host mice also were given CD11b⁺F4/80⁺ macrophages (2 × 10⁶ per host) prepared from naive B6 mice or B6 mice that were presensitized with BALB/c cells. In some experiments, the host mice received injections of 40 × 10⁶ T cells from BALB/c mice and 4 × 10⁶ CD4⁺ T cells from syngeneic B6 mice to prime host macrophages. The severity of GVHD, as assessed by weight loss, was monitored and shown.

Statistical analysis

Mann–Whitney nonparametric statistics were used to determine the level of significance among groups, and a p value < 0.05 was considered significant.

Results

NK cells in naive hosts reject allogeneic nonself via missing self recognition

We labeled allogeneic BALB/c (H-2b) and syngeneic B6 (H-2b) spleen cells with different molar concentrations of CFSE, mixed them at 1:1 ratio, and injected them into naive B6 hosts (H-2b). The survival of CFSE-labeled cells was examined 6–16 h later by
FACS. As shown in Fig. 1A, the allogeneic BALB/c cells were mostly rejected in B6 hosts, whereas the survival of syngeneic B6 cells was not affected. The depletion of NK cells from B6 hosts prevented the rejection of BALB/c cells, and both the labeled BALB/c cells and the B6 cells survived equally well in the NK cell-depleted hosts, confirming that NK cells are the effector cells mediating the killing of allogeneic BALB/c cells (9). This effect requires perforin and is associated with NK cell degranulation, as revealed by staining for CD107a expression (Fig. 1B). In this in vivo cytotoxic assay, the killing of allogeneic cells by host NK cells was ~50% within 16 h of alloantigen exposure (Fig. 1C).

**Rejection of allogeneic cells in presensitized hosts is NK cell independent**

In this strain combination, donor BALB/c cells express H-2Dd, which is the ligand for the NK cell-activating receptor Ly-49D in B6 hosts (22). To examine whether NK cells can be sensitized by donor Ags in vivo, a phenomenon that has been described in other models (12, 23), we challenged B6 mice with BALB/c spleen cells first (priming phase), and 14 d later we performed the same in vivo killing assay as described above (assay phase) to assess whether NK cells exhibit features of memory against the same donor Ags. As compared with that in naive hosts (~50% killing), much greater killing of donor BALB/c cells (but not syngeneic B6 cells) indeed was observed in the presensitization hosts (Fig. 2), and virtually all of the donor cells were killed 16 h later in the sensitized hosts (>95%). Surprisingly, depletion of NK cells from the presensitized hosts was no longer effective in preventing the rejection of donor BALB/c cells, and the donor cells were destroyed completely regardless of NK cell depletion (Fig. 2). NK cell depletion in the primed hosts was confirmed by flow cytometry (data not shown), excluding the possibility of residual NK cells in the killing of donor cells.

One possibility is that, in the primed hosts, T cells, especially CD8+ T cells, may become cytolytic and then reject allogeneic BALB/c cells despite NK cell depletion. To test this possibility, we presensitized CD4KO and CD8KO mice with donor BALB/c cells. NK cells were depleted 14 d later, and then the in vivo killing assay was performed thereafter. Interestingly, we observed that CD8KO mice that were depleted of NK cells still rejected donor BALB/c cells in a fashion similar to that of wild-type B6 mice. However, the rejection of donor BALB/c cells was prevented in NK cell-depleted CD4KO mice (Fig. 2). These

**FIGURE 1.** NK cells promptly kill allogeneic nonself in naive B6 hosts. (A) CFSE-labeled BALB/c (0.2 μM) and B6 (2.5 μM) spleen cells were mixed at a 1:1 ratio and injected into naive C57BL/6 mice with or without NK cell depletion, and a cohort of perforin knockout mice was used as hosts in some experiments. The survival of CFSE-labeled cells in host mice was determined 16 h later by flow cytometry. Data presented are one of three independent experiments. (B) Analysis of CD107a expression by NK cells, a marker of degranulation, with or without a challenge with allogeneic BALB/c cells. Data shown are selectively gated on NK1.1+ cells 5 h after the injection of allogeneic BALB/c cells in vivo. One of three independent experiments is shown. (C) Rejection of allogeneic BALB/c cells in vivo by naive B6, NK cell-depleted B6 mice, and perforin knockout mice 16 h after the injection of CFSE-labeled cells. The bars represent the SDs of three individual samples, and the results shown are representative of three independent experiments.

**FIGURE 2.** Rejection of allogeneic nonself in donor alloantigen-presensitized mice. (A) Wild-type C57BL/6, CD8KO, and CD4KO mice were presensitized with allogeneic BALB/c spleen cells (10^5 per mouse). Two weeks later, NK cells were depleted from these mice (PK136, 0.25 mg/day for 2 d), followed by the transfer of CFSE-labeled syngeneic and allogeneic cells into them to perform the in vivo killing assay. Wild-type C57BL/6 mice presensitized with BALB/c cells without NK cell depletion were used as controls. The survival of CFSE-labeled cells was assessed by flow cytometry 16 h after cell transfer. The plots shown are representative data from one of four experiments. (B) Rejection of allogeneic BALB/c cells by donor Ag-presensitized hosts was calculated. The histograms are mean ± SD of four individual experiments.
FIGURE 3. Rejection of allogeneic nonself in Rag\textsuperscript{−/−}γc\textsuperscript{−/−} hosts passively transferred with different syngeneic and allogeneic cell types. (A) Rag\textsuperscript{−/−}γc\textsuperscript{−/−} mice were left unmanipulated or transferred with allogeneic BALB/c cells (10 \( \times \) 10\(^6\) per mouse), syngeneic CD4\(^+\) T cells (20 \( \times \) 10\(^6\) per mouse), or both, and the in vivo killing assay was performed 2 wk later using either donor BALB/c cells or third-party C3H cells. Groups of host mice also were treated with a depleting anti-CD4 mAb (clone GK1.5, 0.25 mg/day for 2 d) or with gadolinium chloride (20 mg/kg/day for 3 d) before performing the in vivo killing assay. The survival of CFSE-labeled syngeneic and allogeneic cells in the treated hosts was determined by FACS. The plots shown are representative of one of four independent experiments. (B) Rag\textsuperscript{−/−}γc\textsuperscript{−/−} mice were used as host mice for adoptive cell transfer. Licensed macrophages were prepared from CD11b-GFP-DTR transgenic mice presensitized with BALB/c allogeneic cells for 2 wk, and each host was given 2 \( \times \) 10\(^6\) macrophages via tail vein injection. A cohort of host mice transferred with licensed CD11b-GFP-DTR macrophages also was treated with diphtheria toxin (25 ng/day for 2 d), and the in vivo killing assay was performed thereafter. Data shown are from one of three independent experiments.
findings suggest that, in donor Ag-primed hosts, NK cells and CD8+ T cells are both dispensable for the rejection of donor cells upon rechallenge but that CD4+ T cells somehow are required.

**Macrophages act as effector cells in the rejection of allogeneic cells in presensitized hosts**

To dissect whether CD4+ T cells reject donor BALB/c cells directly or indirectly via the activation of other cell types in presensitized hosts, we performed a series of adoptive transfer experiments using Rag-\(^{-/-}\)-ye/c-/- mice as host mice (B6 background, H-2\(^{b}\)); these mice have ample myeloid cells but are deficient for T, B, and NK cells (24). As shown in Fig. 3 and consistent with our published data (9), both BALB/c and B6 cells survived equally well in the Rag-\(^{-/-}\)-ye/c-/- hosts, as assessed by the in vivo killing assay. Next, we adoptively transferred syngeneic B6 CD4+ T cells into Rag-\(^{-/-}\)-ye/c-/- mice first and then performed the in vivo killing assay 14 d later. As shown in Fig. 3A, no killing of donor cells was observed under this condition, suggesting that CD4+ T cells alone do not kill donor BALB/c cells directly. In a different setting in which syngenic B6 CD4+ T cells and donor BALB/c cells were transferred at the same time into Rag-\(^{-/-}\)-ye/c-/- hosts (priming phase), 14 d later we performed the in vivo killing assay to assess the rejection of donor BALB/c cells. As shown in Fig. 3A (upper panel), donor BALB/c cells were rejected completely by Rag-\(^{-/-}\)-ye/c-/- hosts, whereas syngenic B6 cells survived. The depletion of CD4+ T cells from the T cell-transferred Rag-\(^{-/-}\)-ye/c-/- hosts at the time of performing the in vivo killing assay did not affect the rejection of donor BALB/c cells (Fig. 3A, lower panel), supporting the notion that CD4+ T cells do not reject the donors directly. Interestingly, reconstituting Rag-\(^{-/-}\)-ye/c-/- hosts with syngenic B6 CD4+ T cells and donor BALB/c cells did not result in the rejection of third-party C3H cells (H-2\(^{b}\)), demonstrating a degree of donor Ag specificity in this model.

Because Rag-\(^{-/-}\)-ye/c-/- mice are deficient for T, B, and NK cells (24), the depletion of transferred CD4+ T cells at the assay phase did not affect the rejection of donor BALB/c cells. This prompted us to examine whether macrophages (the only major immune cells in these mice) play a significant role in this model. As shown in Fig. 3A (lower panel), in Rag-\(^{-/-}\)-ye/c-/- hosts that were transferred with B6 CD4+ T cells and BALB/c spleen cells, treatment with gadvolinium to inhibit macrophages before the killing assay indeed markedly inhibited the rejection of donor BALB/c cells, and when compared with the untreated controls, a significant fraction of donor BALB/c cells survived in gadolinium-treated Rag-\(^{-/-}\)-ye/c-/- hosts. These findings suggest a key role for macrophages in the rejection of allogeneic BALB/c cells in vivo.

To further ascertain this notion, we repeated the same killing assay in Rag-\(^{-/-}\)-ye/c-/- hosts in which macrophages from donor Ag-presensitized CD11b-GFP-DTR transgenic mice were adaptively transferred (licensed macrophages). As shown in Fig. 3B, both syngenic B6 and allogeneic BALB/c cells survived equally well in Rag-\(^{-/-}\)-ye/c-/- hosts, and transferring the licensed CD11b-GFP-DTR macrophages resulted in the rejection of allogeneic BALB/c cells. The treatment of host mice with diphtheria toxin at the assay phase to eliminate macrophages rescued allogeneic BALB/c cells from being rejected in vivo, providing definitive proof that macrophages can recognize and reject allogeneic cells upon donor Ag presensitization.

**Macrophage-mediated rejection of allogeneic nonself requires CD4+ T cell help**

Clearly, macrophages are capable of rejecting allogeneic nonself, but this response requires donor Ag priming and host CD4+ T cells. To determine the role of CD4+ T cells in this response, we challenged the Rag-\(^{-/-}\)-ye/c-/- hosts with allogeneic BALB/c cells with or without syngeneic CD4+ T cells and determined the macrophage phenotypes 2 weeks later. Macrophages were identified by the expression of F4/80. As shown in Fig. 4, among the cell surface markers examined, CD40 expression was upregulated noticeably after donor Ag priming, whereas other markers did not show marked differences. Because activated CD4+ T cells express CD40L (CD154) (25), one possibility is that activated CD4+ T cells may engage CD40 on macrophages to trigger macrophage activation, which allows macrophages to be alloreactive. To test this possibility, we again transferred allogeneic BALB/c cells and syngeneic CD4+ T cells together into Rag-\(^{-/-}\)-ye/c-/- hosts; the host mice then were treated with anti-CD154 mAb to block CD40/CD40L interactions. Two weeks later, we performed the in vivo killing assay to assess the rejection of CFSE-labeled allogeneic BALB/c cells. As shown in Fig. 5A, the CFSE-labeled BALB/c cells survived in unmanipulated Rag-\(^{-/-}\)-ye/c-/- hosts but were rejected completely in Rag-\(^{-/-}\)-ye/c-/- hosts previously transferred with BALB/c cells and B6 CD4+ T cells. Interestingly, the rejection of BALB/c cells was inhibited completely by blocking the CD40/CD40L pathway (Fig. 5A). Similarly, transferring CD4+ T cells from CD40L knockout mice (CD40L-deficient T cells) to Rag-\(^{-/-}\)-ye/c-/- mice failed to trigger the rejection of allogeneic BALB/c cells upon donor Ag priming (Fig. 5A), demonstrating a critical role for CD40/CD40L interactions in the rejection of allogeneic cells by macrophages. Importantly, the treatment of Rag-\(^{-/-}\)-ye/c-/- hosts (transferred with CD4+ T cells and primed with allogeneic BALB/c cells) with anti-CD154 at the time of performing the killing assay did not prevent the rejection of allogeneic BALB/c cells (Fig. 5B). Thus, CD40/CD40L interactions at the priming phase are required to drive macrophages to an alloreactive mode.

To further examine the role of CD40 in macrophage activation, we primed Rag-\(^{-/-}\)-ye/c-/- mice with allogeneic BALB/c cells, and instead of transferring syngeneic CD4+ T cells at the time of priming, we injected the hosts with an agonist anti-CD40 mAb (FGK45.5) to specifically engage CD40 on macrophages. Rag-\(^{-/-}\)-ye/c-/- mice given the agonist anti-CD40 mAb (FGK45.5) without allogeneic BALB/c cells were included as controls. Two weeks later, the in vivo killing assay was performed to assess the rejec-

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**FIGURE 4.** The phenotypes of macrophages challenged with or without allogeneic BALB/c cells and syngeneic CD4+ T cells in vivo. Rag-\(^{-/-}\)-ye/c-/- mice were transferred with 20 \() mAbs directed against F4/80 and various other cell surface molecules, and the expression of such molecules was analyzed by flow cytometry by gating onto the F4/80+ macrophages. Cells from naive Rag-\(^{-/-}\)-ye/c-/- mice were processed in an identical way and used as controls. The plots shown are representative of one of three independent experiments.
tion of allogeneic BALB/c cells. As shown in Fig. 5C, the stimulation of the CD40 receptor at the time of alloantigen priming triggered the rejection of allogeneic BALB/c cells, whereas CD40 engagement alone failed to do so. Additionally, the treatment of host mice with the agonist anti-CD40 mAb alone did not induce the rejection of BALB/c cells in donor Ag-primed Rag2/2 mice, as described above, MR1 was given at the time of performing the killing assay 2 wk after the priming phase, and the survival of CFSE-labeled indicator cells was assessed by FACS. (C) Rag2/2 mice were transferred with allogeneic BALB/c cells only. The host mice were treated with an agonist anti-CD40 (clone FGK45.5) or a control IgG (0.1 mg i.p.) at the time of donor Ag priming. Some hosts received the agonist anti-CD40 only without allogeneic BALB/c cells. Two weeks later, the in vivo killing assay was performed, and the survival of CFSE-labeled indicator cells was assessed by FACS. All of the results shown in this figure are representative data from three independent experiments.

**Macrophages reject allogeneic nonself cells via phagocytosis**

To explore the mechanisms by which macrophages reject allogeneic cells, we again transferred Rag2/2 mice with syngeneic B6 CD4+ T cells and allogeneic BALB/c cells and treated with anti-CD154 (clone MR1) at 0.25 mg for 2 d or a control IgG. Groups of Rag2/2 mice were transferred with syngeneic CD4+ T cells deficient for CD40L plus allogeneic BALB/c cells. The in vivo killing assay using CFSE-labeled indicator cells was performed 2 wk later, and the killing of CFSE-labeled syngeneic and allogeneic indicator cells was shown. (B) Rag2/2 mice were challenged with allogeneic BALB/c cells and syngeneic CD4+ T cells, as described above, MR1 was given at the time of performing the killing assay 2 wk after the priming phase, and the survival of CFSE-labeled indicator cells was assessed by FACS. (C) Rag2/2 mice were transferred with allogeneic BALB/c cells only. The host mice were treated with an agonist anti-CD40 (clone FGK45.5) or a control IgG (0.1 mg i.p.) at the time of donor Ag priming. Some hosts received the agonist anti-CD40 only without allogeneic BALB/c cells. Two weeks later, the in vivo killing assay was performed, and the survival of CFSE-labeled indicator cells was assessed by FACS. All of the results shown in this figure are representative data from three independent experiments.

We then determined whether licensed macrophages reject allogeneic cells by phagocytosis. To this end, Rag2/2 mice were presensitized with BALB/c alloantigens with or without B6 CD4+ T cells. Naive Rag2/2 mice were used as a control. Two weeks later, all of these mice were injected i.v. with CFSE-labeled allogeneic BALB/c spleen cells. Macrophages were isolated 5 h later, further purified by MACS, and then labeled with...
PE-Cy5-anti-F4/80. The phagocytosis of CFSE-labeled cells was determined by both flow cytometry and confocal microscopy. In this assay, the ingestion of CFSE$^+$ cells by macrophages can be detected by a shift in fluorescence intensity (Fig. 6A). As shown in Fig. 6B, only donor Ag-primed and CD4$^+$ T cell-activated macrophages (licensed macrophages) phagocyted allogeneic BALB/c cells. These data suggest that the rejection of allogeneic cells by macrophages is by phagocytosis rather than by direct killing.

License macrophages exhibit potent regulatory functions in vivo

We used an acute GVHD model to determine whether licensed macrophages exhibit an immunoregulatory role in vivo by eliminating donor T cells. We injected Rag$^{-/-}$ mice with BALB/c T cells to induce acute GVHD responses, and groups of recipient mice also received licensed macrophages from donor Ag-primed B6 mice or macrophages from naive B6 mice. The development of GVHD responses was monitored by changes in body weight and by tissue histology. As shown in Fig. 7, the injection of Rag$^{-/-}$ mice with allogeneic BALB/c T cells induced significant weight loss, and this is associated with extensive cellular infiltration in the intestine (data not shown). This effect was ameliorated significantly in recipient mice transferred with licensed macrophages, whereas macrophages from naive B6 mice did not show any protective effects.

Discussion

In this report, we provide further evidence supporting the presence of an allore cognition system on macrophages, as recently proposed by Zecher et al. (13). We extended those initial observations and further demonstrated that macrophages can recognize and reject allogeneic and that this response requires donor Ag priming and help from activated T cells. The activated and T cell-helped macrophages (licensed macrophages) are surprisingly potent in their rejection of allogeneic nonself in vitro and in their protection against T cell-mediated acute GVHD in vivo. We also showed that the rejection of allogeneic nonself by licensed macrophages is mediated primarily by phagocytosis and that the help delivered by activated T cells is mainly through the CD40/CD40L pathway. These findings, together with findings from other groups (14–16), highlight the importance of macrophages in alloimmunity, a feature that is not formally attributed to them, and the complexity of their interactions with other cell types in the alloimmune response.

A significant finding of our study is that macrophages can be driven to an alloreactive mode in which they recognize and reject allogeneic nonself in an Ag-specific manner. In this mode, macrophages are surprisingly potent in their rejection of allogeneic nonself cells in vivo. This process requires two signals. Specifically, the engagement of alloantigens by macrophages is required but is insufficient alone to mediate the alloreactivity of macrophages. T cell help also is required, and we demonstrate that T cell help is mediated primarily through the CD40/CD40L pathway. Both signals need to be delivered simultaneously to macrophages to license macrophages to respond to allogeneic nonself. Another unexpected feature is that licensed macrophages exhibit donor Ag specificity in that macrophages sensitized against allogeneic BALB/c cells do not show reactivity to third-party C3H cells upon rechallenge. It is unlikely that this type of specificity is provided by T cells, despite the fact that T cell help is required, because an agonist anti-CD40 mAb can replace activated T cells in driving activated macrophages to the alloreactive mode. Although exciting, the molecular nature of such alloreognition remains unknown and is not addressed in the current study. But our findings are reminiscent of those reported by others in a xenogenic islet transplant model (14). In the xenogenic model, mouse macrophages are remarkably potent in rejecting porcine islet transplants; this effect also requires the priming of mouse macrophages by porcine islets and the presence of activated CD4$^+$ T cells at the time of priming.

**FIGURE 6.** Licensed macrophages reject allogeneic cells via phagocytosis. Groups of Rag$^{-/-}$ mice were transferred with 20 × 10$^6$ syngeneic C57BL/6 CD4$^+$ T cells with or without 10 × 10$^6$ allogeneic BALB/c cells. Naïve Rag$^{-/-}$ mice were used as a control. Two weeks later, all of these mice were injected i.v. with 5 × 10$^6$ CFSE-labeled BALB/c spleen cells, and 5 h later macrophages were isolated from the host liver, enriched by MACS, and further stained with PE-Cy5-anti-F4/80. The cell preparation was analyzed by flow cytometry (A) and fluorescence microscopy (B). Phagocytosis was identified by a shift in fluorescence intensity in FACS plots or the appearance of yellow cells under the fluorescence microscope. Original magnification ×200. The results shown are representative of three independent experiments.

**FIGURE 7.** Licensed macrophages exhibit potent regulatory function in vivo in an acute GVHD model. Groups of male Rag$^{-/-}$ mice were given 40 × 10$^5$ BALB/c T cells via tail vein injection, and mice then were followed for signs of GVHD. The severity of GVHD was assessed by weight loss over a time period of 3 wk. Some mice received additional CD11b$^+$ macrophages (2 × 10$^6$ per mouse) purified from naive B6 mice or B6 mice presensitized with BALB/c cells for 2 wks or syngeneic B6 CD4$^+$ T cells. Changes in body weights among different groups were monitored. Values are mean ± SD of five mice at individual time points, and one of two sets of experiments is shown. (*p < 0.05, **p < 0.01).
Importantly, porcine Ag-primed and CD4+ T cell-activated macrophages, upon transfer into immunodeficient NOD/SCID hosts, induce prompt rejection of porcine islet xenografts, whereas unprimed macrophages or those primed without CD4+ T cells fail to do so. Additionally, macrophages primed with porcine xenoantigens (along with activated CD4+ T cells) fail to reject rat islet xenografts or mouse islet allografts (14). Collectively, these data provide compelling evidence that macrophages are capable of recognizing allotransplants in an Ag-specific manner.

The exact molecular nature of this macrophage allorecognition system remains to be defined. But there are several possibilities that may explain this phenomenon. Macrophages are known to express the inhibitory receptor Sirpα, which binds to its ubiquitously expressed ligand CD47 (26). Thus, CD47/Sirpα is an important inhibitory pathway in suppressing macrophage activation, and species incompatibility between CD47 and Sirpα often triggers the rejection of xenogenic cells by macrophages (27). But the role of this pathway in the allogeneic setting is less clear. CD47 is expressed ubiquitously by all tissues and cells, and it is not shown to have allospecificity in the mouse (26). Also, macrophages express an extensive array of pattern recognition receptors, including TLRs, that recognize conserved structures from bacteria and viruses (28); these receptors also may recognize conserved residues in molecules in the mouse. Some of these receptors are known to respond to endogenous danger signals or inflammatory ligands, such as high mobility group box 1 and uric acid (29). However, such ligands are highly conserved and should be shared between self and nonself tissues and cells. Alternatively, the specificity of licensed macrophages may have been imprinted at the time of alloantigen priming (like the homing properties of certain cell types), but this possibility remains to be tested. Considering the inducible nature of this allorecognition system as shown in our study, this allorecognition system may be quite different from that described in other settings (14). This is a significant issue that warrants further investigation.

There are several mechanisms by which macrophages damage or kill target cells, and such mechanisms include the production of cytotoxic or cytostatic cytokines and ROS and the rejection of target cells by phagocytosis (27). It is not known how licensed macrophages chose one over another, but different effector mechanisms likely are activated by different pathways or under different contexts. In our study, phagocytosis seems to be the primary effector mechanism in vivo in the rejection of allogeneic cells, because licensed macrophages in our study do not produce ROS, nor do they express typical cytolytic molecules. However, the in vivo situation is far more complex; the possibility of a multiplicity of mechanisms involved in protection against GVHD cannot be excluded.

Our data expand the traditional role of macrophages as inflammatory cells or APCs and further suggest that macrophages can be directly alloreactive in transplant settings. Clearly, the elimination of allogeneic T cells by licensed macrophages in a GVHD setting is desirable and beneficial to the hosts. However, in solid organ transplantation, graft-infiltrating macrophages, once becoming alloreactive, may contribute significantly to graft damage and poor transplant outcomes. Considering the importance of CD40/CD40L interactions in licensing macrophages, cell types that can express CD40L may have the potential to drive macrophages to an alloreactive mode, and in addition to activated T effector cells, memory T cells, activated endothelial cells, or even platelets, all express CD40L (30–32) and therefore may act as key partners in macrophage-mediated rejection. This may help to explain why allografts with ample infiltrating macrophages tend to have poor outcomes in response to conventional immunosuppression therapies (17). Our data also suggest that the tolerogenic effects of anti-CD154 mAb in transplant survival may extend to the inhibition of macrophage licensing, in addition to blocking T cell costimulation. In conclusion, our data suggest that macrophages can be driven to an alloreactive mode and that this process requires donor Ag priming and help from activated T cells. Importantly, our data support the notion that macrophages express an allore cognition system that allows macrophages to respond to allografts under certain conditions (13, 14). A better appreciation of this system may have important clinical implications.

Acknowledgments
We thank members of the Li laboratory for countless discussions and Alice E. Li at ASAP Editing for help with editing the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References