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ARTICLE

Primed macrophages directly and specifically reject allografts

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Monocytes and macrophages have long been associated with acute and chronic allograft rejection; this is mediated by their abilities to promote inflammation, kill target cells via antibody-dependent cytotoxicity and modulate adaptive immunity. Our present study showed that allogeneic antigen-primed macrophages acutely rejected skin grafts with specificity after adoptive transfer into MHC-matched immunodeficient mice. The ability of primed macrophages to reject allografts essentially requires the help of $CD4^+$ T cells and does not require the help of $CD8^+$ T cells. Moreover, the primed, perforin-deficient macrophages rejected the skin grafts in a significantly delayed pattern compared with WT macrophages, indicating that the perforin pathway of the primed macrophages is likely involved in the rejection process. Thus, primed macrophages are endowed with adaptive immunity-like features, such as specificity, with the help of $CD4^+$ T cells during the immune response to allografts. The present study challenges our traditional views of macrophage functions and highlights the biological functions of macrophages beyond innate immunity in mammals.

Key words: Macrophages; Specificity; Graft rejection; Innate immunity; Transplantation

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INTRODUCTION

Both innate and adaptive immune cells are closely involved in host defense against pathogens, tissue damage in autoimmunity and transplanted organ rejection. Innate immunity is an evolutionarily conserved response capable of fighting diverse pathogens, even in plants and invertebrate animals.[1] Adaptive immunity developed much later and evolved over time by a gene conversion mechanism; this led to lymphocyte receptors and the subsequent genomic invasion by retroposon encoding site-specific recombinases,[2] which generate unlimited numbers of receptors to allow the adaptive immune cells to specifically recognize antigens. It is generally believed that innate immune cells lack antigen-specific properties, which contrasts with adaptive immune cells. However, this belief was recently challenged by observations showing the antigen-specificity properties of innate immune cells, especially those of NK cells.[3–7] Monocytes/macrophages, one of the key elements of the innate immune system, are critical effectors in tissue inflammation and are the first line of defense of the immune system. It has long been recognized that monocytes/macrophages are associated with acute and chronic allograft rejection,[8,9] mainly through their ability to promote inflammation, kill target cells via antibody-dependent cytotoxicity and modulate adaptive immunity.[10,11] However, whether innate macrophages in mammals have the potential to mediate specific immune responses as effector cells remains an open, fundamental question. In the present study, we employed an allogeneic skin-grafted immunodeficient mouse model with the adoptive transfer of MHC-matched macrophages to address this issue.

MATERIALS AND METHODS

Mice

Six- to eight-week-old C57BL/6 (B6, H-2^b), BALB/c (H-2^d), C3H (H-2^k), SJL (H-2^S), and FvB (H-2^q) mice were purchased from Vital River Laboratories (Beijing, China). Six- to eight-week-old severe combined immunodeficient (SCID, H-2^d) and Rag2 KO (H-2^b) mice were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). Perforin KO mice (H-2^b) were kindly provided by Professor Lianfeng Zhang at the Institute of Laboratory Animal Science at the Chinese Academy of Medical Sciences and Peking Union Medical College. CD45.1 (B6, H-2^b) mice were purchased from the Department of Laboratory Animal Science at the Peking University Health Science Center (Beijing, China). All mice were maintained in a specific, pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals by the Institute of Zoology, CAS (Beijing, China).

Reagents

The following mAbs were purchased from eBioscience: fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse F4/80 mAb (BM8; IgG2a), phycoerythrin-Cy5 (PE-Cy5)-conjugated rat anti-mouse CD11b mAb (M1/70; IgG2b), PE-conjugated Rat anti-mouse B220 (RA3-6B2; IgG2a), PE-conjugated American hamster anti-mouse TCRβ mAb (H57-597; IgG), and PE-conjugated rat anti-mouse CD45 (MA5-17963; IgG1). The following mAbs were purchased from Biolegend: PE-Cy5-conjugated rat anti-mouse CD8a mAb (53-6.7;

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IgG2a), PE-conjugated rat anti-mouse CD8a mAb (OX-8; IgG1), FITC-conjugated mouse anti-mouse granzyme B mAb (GB11; IgG1), and PE-conjugated rat anti-mouse CD49b mAb (DX5; IgM). The following mAbs were purchased from BD Biosciences Pharmingen (San Diego, CA, USA): PE-conjugated rat anti-mouse CD4 mAb (GK1.5; IgG2b), PE-conjugated rat anti-mouse Ly6G mAb (1A8; IgG2a), PE-conjugated American hamster anti-mouse CD11c mAb (HL3; IgG1), and FITC-conjugated mouse anti-mouse CD45.1 mAb (A20, IgG2a). A rabbit anti-mouse perforin mAb (#3693) was purchased from Cell Signaling Technology. A goat anti-mouse granzyme B mAb (AF1865) was purchased from R&D. A rat anti-mouse F4/80 mAb was purchased from Abcam. A rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by a 2.4G2 hybridoma (ATCC, Rockville, MD, USA) in our laboratory. A PE mouse anti-Ki-67 antibody set (Cat 556027) was purchased from BD Bioscience Pharmingen.

Preparation of peritoneal macrophages

Mouse peritoneal exudate cells were obtained from the peritoneal exudates of mice.[12] For adoptive transfer to immunodeficient mice, the peritoneal cells were stained with F4/80-FITC, CD11b-PE-Cy5, CD4-PE, CD8-PE, B220-PE, Ly6G-PE, CD11c-PE, and CD49b-PE at 4 °C for 30 min, and then the $CD4^-CD8^-B220^-Ly6G^-CD11c^-CD49b^-F4/80^+CD11b^+$ peritoneal macrophages (PEMs) were sorted by flow cytometry (99% were PEMs). The sorted macrophages were adoptively transferred to MHC-matched SCID or syngeneic Rag2 KO mice via the tail vein ($0.5–1 \times 10^6$ per mouse for detecting the rejection of allo-skin grafts or $1.5–2 \times 10^6$ per mouse for detecting the transferred macrophages in recipients). For ex vivo experiments, peritoneal cells were washed twice with cold Hanks' solution, and these cells were adjusted to 1×10^6 cells/ml in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) and cultured in 24-well plates (Costar, Cambridge, MA, USA) for 2 h at 37 °C and 5% CO_2. The non-adherent cells were removed by washing them with warm RPMI 1640 medium. The adherent cells were harvested with ice-cold PBS (pH 7.2) and readjusted to 5×10^5 cells/ml. The cell viability was usually more than 95%, as determined by trypan blue exclusion. The adherent cells constituted more than 90% of $F4/80^+$ macrophages, as reported previously.

Adoptive transfer of $CD4^+$ T cells or $CD8^+$ T cells

In some experiments, the splenocytes from BALB/c mice were stained with CD4-PE and CD8-PE-Cy5. The cells were sorted by a MoFlo XDP sorter (Beckman), and 2×10^6 sorted BALB/c $CD4^+$ or $CD8^+$ T cells were adoptively transferred to MHC-matched immunodeficient SCID mice via the tail vein.

Flow cytometry

Peritoneal macrophages were washed once with FACS buffer (PBS, pH 7.2, containing 0.1% NaN_3 and 0.5% bovine serum albumin).[13] To detect the effector molecules, the macrophages were harvested after stimulation with allo-splenocytes and then were stained with a PE-Cy5-labeled anti-mouse F4/80 mAb. The cells were subsequently fixed and analyzed for the intracellular production of cytokines (BD Pharmingen) by staining with FITC-labeled granzyme B, perforin or the isotype control Ab. The peripheral blood, splenocytes, and liver cells that were harvested after RBC removal were stained with CD45.1-FITC or TCRβ-PE. The cells from the harvested skin grafts were stained with CD45.1-FITC and CD45-PE. Cell proliferation assays with Ki-67 were performed according to the protocol of the PE mouse anti-Ki-67 set (BD Bioscience Pharmingen, 556027). Nonspecific FcR binding was blocked by an anti-mouse FcR mAb 2.4G2. At least 10,000 cells were assayed by FCM using a Beckman Coulter Epics XL benchtop flow cytometer, and the data were analyzed with CXP v2.2 software (Beckman Coulter).

Stimulation of macrophages with allogeneic cells

Single-cell suspensions of allogenic splenocytes were freshly obtained and cultured in RPMI 1640 medium in six-well plates (Costar, Cambridge, MA, USA) for 2 h at 37 °C and 5% CO_2. Then, the non-adherent splenocytes were harvested and added to the prepared macrophages at a ratio of 5:1 for 1 h at 37 °C and 5% CO_2 in 48-well plates. The stimulating splenocytes were removed by washing them with warm RPMI 1640 medium, and the adherent macrophages were harvested for FCM, Western blot, immunofluorescence or real-time PCR.

Skin transplantation

Skin grafts from BALB/c, C57BL/6 or the 3rd party (such as FvB) mice were transplanted into SCID or Rag2 mice as described previously.[14] For skin transplantation, erythema, edema, and hair loss were considered early signs of rejection, whereas ulceration, progressive shrinkage, and desquamation were considered to be the end point of rejection. Photographs were taken every two days with a digital camera (Canon EOS450D; Canon, Tokyo, Japan) until the graft was rejected completely. The skin grafts were removed on the fifth day of transplantation and rinsed in cold PBS, placed in OCT compound for immunofluorescence assessment.

Detection of adoptively transferred macrophages in skin grafted recipients

The sorted macrophages from unimmunized or BALB/c splenocyte-immunized CD45.1 B6 mice were adoptively transferred ($1.5–2 \times 10^6$ per mouse) to T and B-deficient Rag2 KO mice via the tail vein before grafting the allogeneic BALB/c skin (Day 0). At Day 15, the peritoneal cells, peripheral blood, and splenocytes from skin-grafted Rag2 KO mice were harvested after RBC removal (for peripheral blood and splenocyte collection); these cells were stained with CD45.1-FITC following detection by FCM. The sorted $CD45.1^+$ macrophages from the splenocytes were used for RNA extraction and real-time PCR assays.

Preparation of cells from skin grafts and livers

Skin grafts from SCID mice or Rag2 KO mice were isolated and removed from the subcutaneous fat tissues, and the livers were first perfused with prewarmed PBS to remove RBCs via the portal vein. Skin grafts and livers were cut to 0.5–1 cm^2 squares after rinsing with PBS thoroughly. Then, pieces of skin grafts and livers were digested in serum-free RPMI 1640 medium with collagenase type IV (Sigma-Aldrich, 1 mg/m) and DNase I (Sigma-Aldrich, 0.01 mg/ml) at 37 °C for 30 min, followed by washing with RPMI 1640 medium and filtration through a 50-mM nylon filter to obtain single cells. Some samples used for RNA extraction were stained with CD45.1-FITC for the flow cytometry assay.

Western blot

Macrophages stimulated with allo-splenocytes and IL-2 (10 U/ml) for 4 h were lysed with RIPA-containing proteinase inhibitor (Roche, Cat 04693116001) and then boiled with 2x SDS loading buffer; this was followed by assays with rabbit anti-mouse perforin (CST, #3693) or sometimes with the additional goat anti-mouse granzyme B (R&D, AF1865) through western blots.[15] β-actin (Cell Signaling) was quantified as a loading control.

Immunofluorescence

For immunofluorescence, the stimulated PEMs or serial sections (5 mm) of skin grafts from the OCT-embedded frozen tissues were fixed in cold acetone and were blocked in PBS/1% BSA. Then, the tissues were washed in PBS/0.05% Tween 20 and were incubated with the optimal antibody dilutions of anti-F4/80, anti-granzyme B or anti-perforin for 1.5 h at room temperature. The tissues were washed and incubated with the appropriate, following secondary reagents: Alexa Fluor 546 goat anti-rat IgG (H+L), Alexa Fluor 488

goat anti-rabbit IgG (H+L) (Invitrogen), and Alexa Fluor 488 donkey anti-goat IgG (H+L) (Santa Cruz). The control slides were incubated with isotype-matched Ig antibodies. The images were acquired with two-photon microscopy (Carl Zeiss).

Quantitative RT-PCR

The RNA was purified from the control and immunization PEMs, which were sorted by flow cytometry and characterized as F4/80$^+$. The mRNA was prepared using an RNeasy Mini kit (Qiagen), and the cDNA library was generated with a reverse transcription system kit (Promega) according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio). The relative expression values of the target genes normalized to HPRT were obtained. The primers used in the current study are listed in Table 1.

Statistical analysis

All data are presented as the mean±SD. Student's unpaired *t*-test for comparison of means was used to compare the groups. A P value <0.05 was considered statistically significant.

RESULTS

Primed macrophages rejected allogeneic skin grafts

It is well known that SCID mice and Rag2 KO mice, which lack both T cells and B cells, fail to reject allogeneic, or even xenogeneic, skin grafts. Thus, it is widely accepted that innate immune effectors, such as macrophages alone, are insufficient to reject allogeneic organ grafts. However, whether macrophages in immunocompetent mice primed with allogeneic antigens can gain the ability to reject allografts has never been investigated. To determine whether the primed macrophages can directly reject allogeneic grafts, we first immunized BALB/c (H-2^d) mice with allogeneic B6 (H-2^b) splenocytes or skin grafts (Fig. 1a). The sorted F4/80$^+$CD11b$^+$CD4$^-$CD8$^-$B220$^-$Ly6G$^-$CD11c$^-$CD49b$^-$ peritoneal macrophages (Supplementary Fig. 1) of the unimmunized BALB/c mice (control) and B6 splenocyte-immunized BALB/c mice (primed) were adoptively transferred into MHC-matched SCID mice (H-2^d). These SCID mice were then grafted with either B6 or the third-party skin from FvB (H-2^q), C3H (H-2^k), and SJL (H-2^s) mice (Fig. 1a). As expected, the control SCID mice that received no macrophages or unprimed macrophages accepted the allogeneic skin grafts for more than 100 days without any signs of rejection due to the absence of T cells (Fig. 1b, c). Surprisingly, SCID mice that received MHC-matched macrophages from B6 splenocyte-immunized BALB/c mice (primed macrophages) efficiently rejected the allogeneic B6 skin grafts within 40 days, and these mice rejected the third-party skin grafts in a significantly slower pattern ($P < 0.001$, Fig. 1b, c). Furthermore, SCID mice that received the isolated MHC-matched macrophages from B6 skin-immunized BALB/c mice also rejected the B6 allo-skin grafts rapidly (Supplementary Fig. 2). To exclude the potential involvement of T cells in the secondary SCID recipients, we also used immunodeficient Rag2 KO mice (H-2^b) as syngeneic recipients. Rag2 KO mice that were adoptively transferred with macrophages from BALB/c splenocyte-immunized B6 mice quickly rejected the allogeneic BALB/c skin grafts (Supplementary Fig. 3). Importantly, there were no detectable TCRβ$^+$ cells in the peripheral blood and spleens of SCID and Rag2 KO mice that received the primed macrophages before or after skin allograft rejection (Fig. 1e, Supplementary Fig. 3), excluding the potential pollution of T cells in these models. Thus, these studies collectively indicate that donor antigen-immunized macrophages in immunocompetent mice can efficiently mediate allogeneic graft rejection in a donor-specific manner after an adoptive transfer to immunodeficient recipients, although a degree of cross-reactions with other allogeneic antigens may exist.

CD4$^+$ T cells are essential for naïve macrophages to gain the ability to reject allografts

Because the naïve macrophages could not reject the allogeneic grafts, we tried to determine whether T cells are essential for the ability to reject allogeneic grafts with primed macrophages during immunization in immunocompetent mice. SCID mice (H-2^d) that received the MHC-matched CD4$^+$ T cells or CD8$^+$ T cells of BALB/c mice (H-2^d) were immunized with B6 skin grafts. We then adoptively transferred the sorted macrophages from these immunized, T cell-reconstituted SCID mice into the secondary SCID mice and subsequently grafted the allogeneic B6 skin onto these mice on the second day (Fig. 2a). SCID mice that received the adoptive transfer of the primed macrophages from the B6 skin-immunized, MHC-matched CD4$^+$ T cell-reconstituted SCID mice efficiently rejected the B6 skin grafts, while the SCID mice that received the sorted macrophages, either from the B6 skin-immunized SCID mice alone or from the B6 skin-immunized, MHC-matched CD8$^+$ T cell-reconstituted SCID mice, failed to reject the B6 skin grafts within 100 days (Fig. 2b, c). Thus, the help of CD4$^+$ T cells but not CD8$^+$ T cells during the priming period is essential for macrophages to gain the ability to reject allografts.

Primed macrophages proliferate during the immune response

After concluding that the primed macrophages (with the help of CD4$^+$ T cells) can gain the ability to reject allografts with a certain degree of specificity, we aimed to further clarify the effector roles of adoptively transferred macrophages in the rejection of allografts. We thus observed the presence of the transferred macrophages in these mice during graft rejection. Rag2 KO mice (CD45.2, H-2^b) were adoptively transferred with macrophages from BALB/c-immunized or unimmunized CD45.1 B6 mice (H-2^b); then, these mice received transplants of allogeneic BALB/c or the third-party FvB skin. After 15 days, we detected the levels of the adoptively transferred CD45.1$^+$ macrophages in the skin-grafted CD45.2 Rag2 KO mice. We found significantly more donor CD45.1$^+$ macrophages in the skin-grafted CD45.2 Rag2 KO mice that received the primed CD45.1$^+$ macrophages than the number of donor macrophages in the mice with the naive CD45.1$^+$

Table 1. Sequencing primers used in the present study

Primers	Sense Sequence (5′→3′)	Antisense Sequence (5′→3′)
Granzyme A	ACCAGGAACCAGATGCCGAGTA	GGCGATCTCCACACTTCTCTCC
Granzyme B	GCTGACTGCTGCTCACTGT	CACATCTCCTGGCTTCACATTG
Granzyme C	TCCTCCATCCTGAGCAGCCTTC	GGAAGCCTCCGCAGAACATCTT
Granzyme D	CATCTCCTTCCTCGCCTTCCAA	TGAGCAGCCGTCAGCACAA
Granzyme E	TGCCACCAGTCCTGATTCTCCT	TGAACCAAGAAGCCTCCACAGT
Granzyme F	TTCCTGTGGAGGCTTCCTGGTT	GCATTGGGTCTGGGCAACTTGA
Perforin	ATCCGACAGTGGCGTCTTGGT	TGACCGAGTGGCAGTGTAGCA

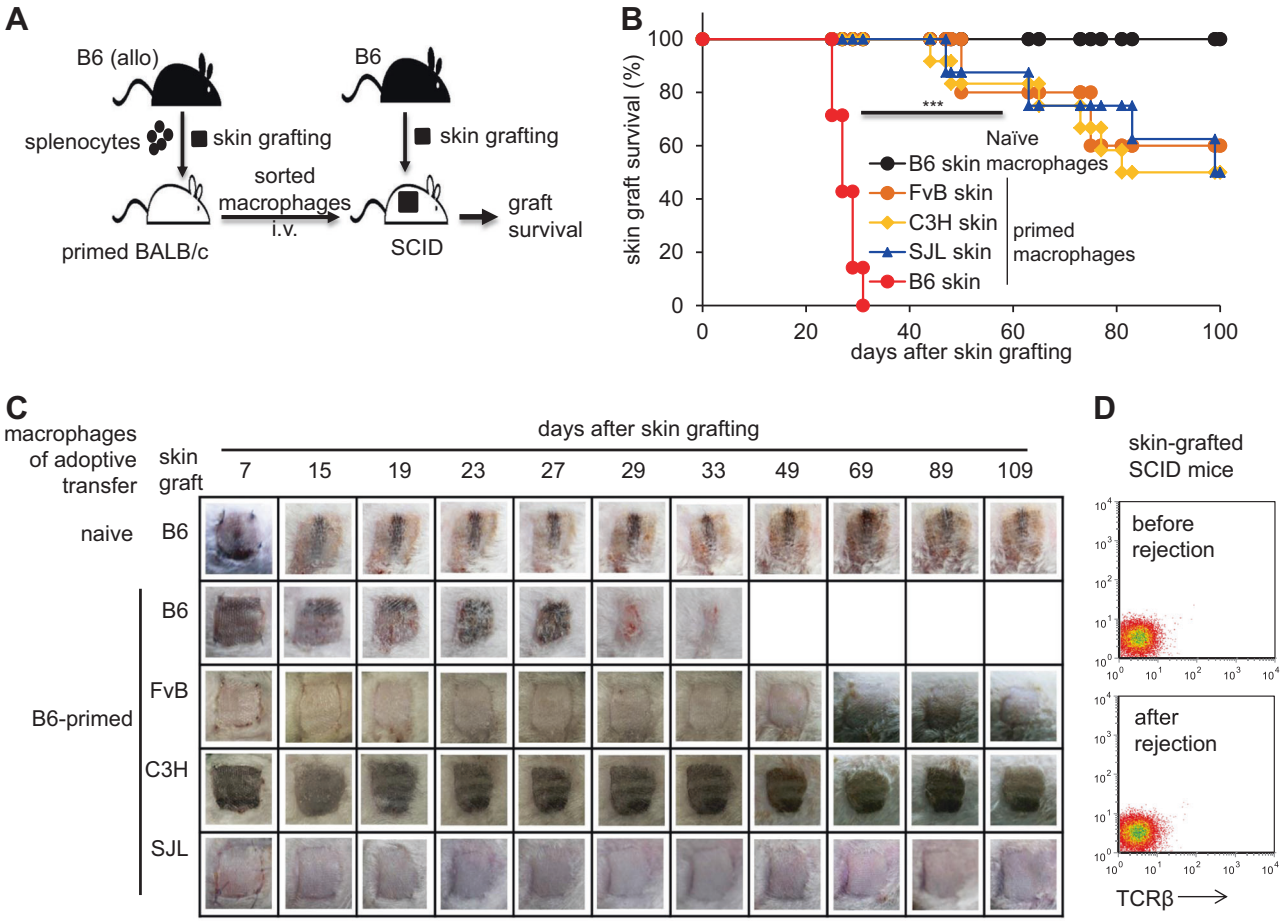

Fig. 1 Primed macrophages reject skin allografts with specificity. BALB/c mice were immunized with B6 splenocytes (1×10^7 cells per mouse) or B6 skin. After 10 days, the sorted peritoneal macrophages from unimmunized or immunized mice were adoptively transferred into immune-deficient SCID mice before skin grafting. **a** The schema of primed macrophage-mediated rejection of skin grafts is shown. **b** The survival curves of allogeneic donor B6 or different third-party skin grafts in SCID mice with the adoptive transfer of the unprimed and B6 splenocyte-primed macrophages are summarized. **c** SCID mice were transplanted with allogeneic donor B6 or different third-party (FvB, C3H and SJL) skin grafts. The macroscopic pictures of allo-skin grafts were taken at different time points. **d** The detection of TCRβ$^+$ cells in the peripheral blood of SCID mice that received the primed macrophages before and after the allo-skin graft rejection. ***$P < 0.001$ compared with the control or the indicated groups

macrophages, as indicated by the high percentages of donor CD45.1$^+$ macrophages found in the blood, spleen, peritoneal cavity and liver (Fig. 3a, b). Importantly, significantly more of the transferred macrophages found in the BALB/c skin grafts in Rag2 KO mice were primed macrophages than those found in the third-party FvB skin grafts, whereas fewer naive macrophages were found in both the BALB/c and FvB skin grafts in the Rag2 KO mice that received naive macrophages ($P < 0.001$, Fig. 3c). These data further indicate that the primed macrophages likely act as effector cells to directly reject allografts. Recent reports have identified that macrophages can accumulate in tissues,[16–19] self-renew[20–22] and undergo significant proliferation in vivo.[17,23,24] We detected macrophage proliferation by Ki-67 staining and found that the primed CD45.1$^+$ macrophages indeed proliferated when they re-encountered allo-antigens in vivo, as assayed 15 days after skin grafting (Fig. 3d). These data provide evidence that the primed macrophages can expand when they re-encounter the priming antigens, similar to the features of T cells[25,26].

The perforin pathway is involved in primed macrophage-mediated allograft rejection

The mechanisms by which the primed macrophages mediate allograft rejection were explored. It was reported that macrophages could reject target tumor cells via the perforin/granzyme B pathway,[27–29] which is usually recognized as the predominate effector pathway employed by NK cells and CD8$^+$ T cells to kill the target cells.[30,31] We asked whether the primed macrophages employ similar strategies to reject allografts. Indeed, the primed F4/80$^+$CD11b$^+$ macrophages (PEMs from B6 mice that were immunized with BALB/c splenocytes for 10 days) express significantly higher granzyme B and perforin levels when rechallenged with donor cells in vitro, as determined by real-time PCR ($p < 0.001$, Fig. 4a), intracellular staining flow cytometry ($p < 0.001$, Fig. 4b–e), Western blots (Fig. 4f) and confocal microscopy (Fig. 4g). Identical to the ex vivo observation, the CD45.1$^+$ macrophages in the Rag2 KO recipients with the sorted primed CD45.1$^+$ macrophages showed significantly higher expression levels of granzyme B and perforin than those in the Rag2 KO mice that received naive macrophages 15 days after skin grafting, as detected by real-time PCR ($p < 0.001$, Fig. 5a). More granzyme B and perforin were expressed in the donor (B6) skin grafts in SCID recipients who were adoptively transferred with the primed F4/80$^+$CD11b$^+$ macrophages compared with those expressed in the donor skin grafts in mice that received the unprimed F4/80$^+$CD11b$^+$ macrophages or those expressed in the third-party (FvB) skin grafts in mice that received the primed F4/80$^+$CD11b$^+$ macrophages ($p < 0.001$, Fig. 5b). These data suggest that granzyme B and perforin may be the effector molecules for

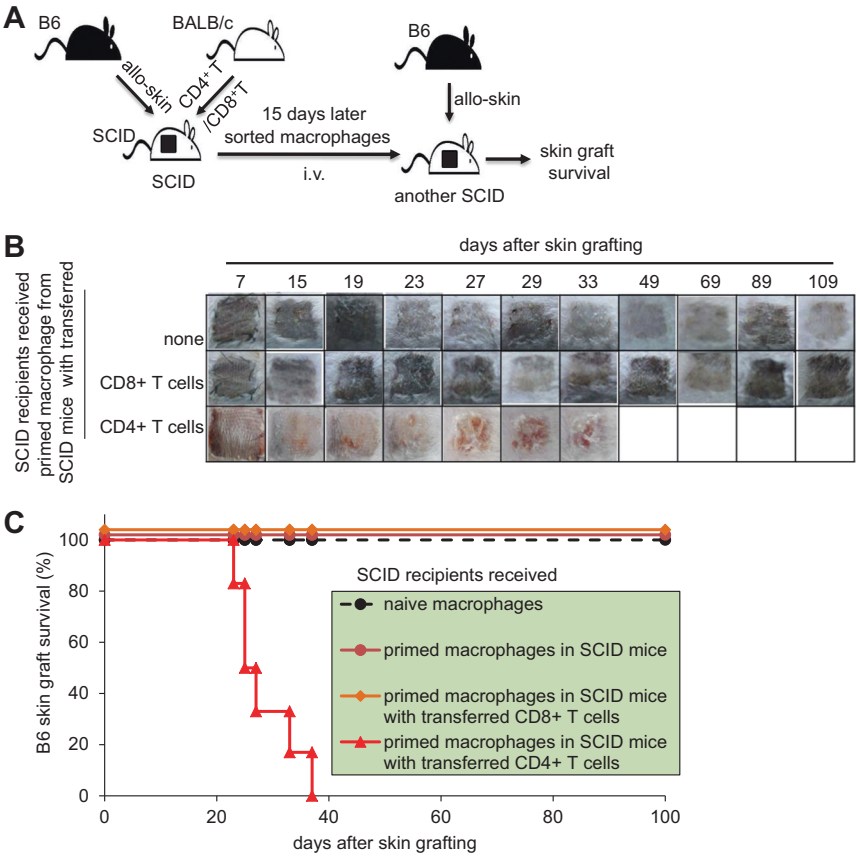

Fig. 2 $CD4^+$ T cells are essential for primed macrophages to gain the ability to reject skin allografts. SCID mice were immunized with allogeneic B6 skin and simultaneously received MHC-matched $CD4^+$ T or $CD8^+$ T cells from BALB/c splenocytes. Fifteen days later, the sorted macrophages from the primed SCID mice were adoptively transferred to other SCID mice (the secondary recipients). These SCID mice were then grafted with allogeneic B6 skin as indicated in the schema **a**. **b** The macroscopic pictures of allo-skin grafts were taken at different time points. **c** The survival curves of the B6 skin allografts in the secondary SCID recipients with the adoptive transfer of B6 skin-primed macrophages of SCID mice alone or in the SCID mice who received the MHC-matched $CD4^+$ T or $CD8^+$ T cells are shown

the primed macrophage-mediated rejection of skin allografts. To confirm this hypothesis, we immunized perforin KO mice (H-2^b) and wild-type (WT) mice with B6 genetic backgrounds (Fig. 6a) with BALB/c skin grafts and then adoptively transferred the sorted macrophages into syngeneic Rag2 KO mice (H-2^b). The splenocytes of the perforin KO mice failed to express the perforin protein even after IL-2 stimulation, which is reported to increase perforin expression,[32,33] whereas the WT splenocytes expressed the perforin protein. The Rag2 KO mice that received the primed perforin KO macrophages significantly delayed the rejection of skin grafts compared with the rejection time of those mice that received the primed WT macrophages ($P < 0.001$, Fig. 6b, c). These results indicate that the primed macrophages reject allografts, at least partially, in a perforin-dependent manner.

DISCUSSION

For the first time, our current study demonstrates that primed macrophages in immunocompetent mice can act as effector cells to directly reject allogeneic grafts in mice via a perforin-dependent pathway; in addition, the gained rejection capability of macrophages requires the help of $CD4^+$ T cells in the priming phase. More importantly, allogeneic, antigen-primed macrophages display specificity properties (one of the adaptive immunity-like features) during allograft rejection. This observation clearly subverts our traditional opinion that macrophages act as part of the innate immune system and will significantly impact our understanding of the biological significance of macrophages beyond innate immunity.

It is traditionally accepted that T cells are both necessary and sufficient for the acute rejection of most allografts; in addition, acute organ allograft rejection has long been attributed to the adaptive immune cells with an inflammatory contribution from the innate immune cells.[34] Although the macrophages of the innate immune system could directly phagocytose allogeneic cells,[35,36] macrophages participate in solid organ allograft rejection mainly through their ability to promote inflammation, kill target cells via antibody-dependent cytotoxicity and modulate adaptive immunity.[10,11] Here, we demonstrate that primed macrophages alone can directly mediate the rejection of allogeneic skin grafts. This conclusion is supported by the following evidence: (1) T and B cell-deficient mice (SCID mice) that received adoptive transfers of primed macrophages from B6 splenocyte- or skin- immunized BALB/c mice could rapidly reject B6 skin grafts, but immunodeficient mice that received unimmunized MHC-matched macrophages could not. (2) The primed macrophages infiltrated the donor skin grafts in SCID mice, and these cells expressed high levels of perforin and granzyme B; however, the unprimed macrophages failed to do so. (3) The primed macrophages, but not the unprimed macrophages, significantly proliferated and increased the cell number in SCID mouse recipients after skin grafting. (4) The T cells are almost undetectable in SCID and Rag2 KO mice, even after allogeneic skin grafting.

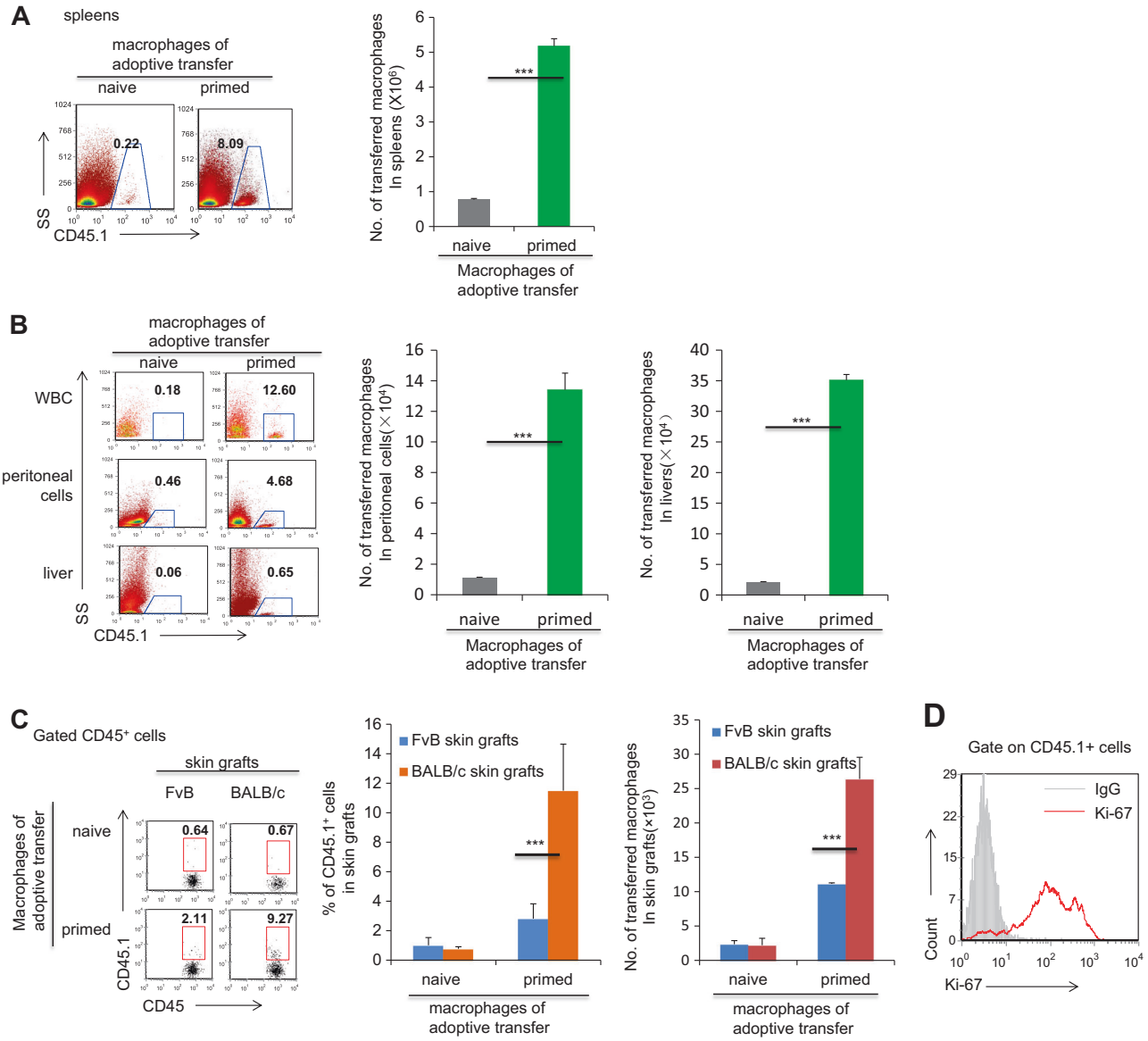

Fig. 3 Primed macrophages proliferate in allo-skin grafted mice. Peritoneal macrophages were collected or sorted from unimmunized CD45.1 B6 mice and from CD45.1 B6 mice immunized with BALB/c splenocytes for 10 days. These sorted macrophages were then adoptively transferred into Rag2 KO mice (CD45.1) before grafting donor BALB/c skin or the third-party FvB skin. The percentages and cell numbers of the transferred CD45.1$^+$ macrophages are detected in the spleen (**a**), peripheral blood, peritoneal cavity, and liver (**b**) and skin grafts 15 days after skin grafting (**c**). **d** FACS staining is shown for Ki-67 in CD45.1$^+$ macrophages in allo-skin grafted recipients received the primed CD45.1$^+$ macrophages. The data are shown as the mean±SD ($n = 3$), which were one representative of two independent experiments. ***$P < 0.01$ compared with the indicated groups

The gained macrophage ability to reject allografts depends on immunization with allogeneic antigens. Additionally, CD4$^+$ T cells but not CD8$^+$ T cells are essential for naïve macrophages to gain this distinguished ability during immunization. This observation is in agreement with previous studies showing that CD4$^+$ T cell-activated macrophages could mediate the rejection of allogeneic cells.[35,37] This phenomenon likely indicated the bidirectional communication between the adaptive immune system's CD4$^+$ T cells and the innate immune system's macrophages during the immune response. In allograft recipients, macrophages promote local inflammation and stimulate the effector T cells; however, it is likely that the CD4$^+$ T cells can simultaneously endow the activated macrophages with the capability to reject allografts as effector cells. Unfortunately, the underlying molecular mechanisms and the crosstalk pathways are unclear and need future clarification.

More importantly, allogeneic antigen-primed macrophages, which mediate allograft rejection, display specificity properties; these specificity properties were traditionally considered to be exclusive features of the adaptive immune system, but this has recently been challenged by recent studies showing the antigen-specific properties of innate immune cells. In animal models of contact hypersensitivity or viral infection, the innate immune system's NK cells could specifically respond to previously encountered antigens.[6,38,39] Our data show that donor antigen-primed macrophages rapidly rejected donor allo-skin grafts but mediated the significantly slower rejection of skin grafts from third-party mice. This cross-reaction in the macrophage-mediated rejection of different skin grafts suggests that the response specificity of the primed macrophages may not be as rigorous as that of T cells; it is also possible that some antigens that were recognized by the primed macrophages were also expressed by

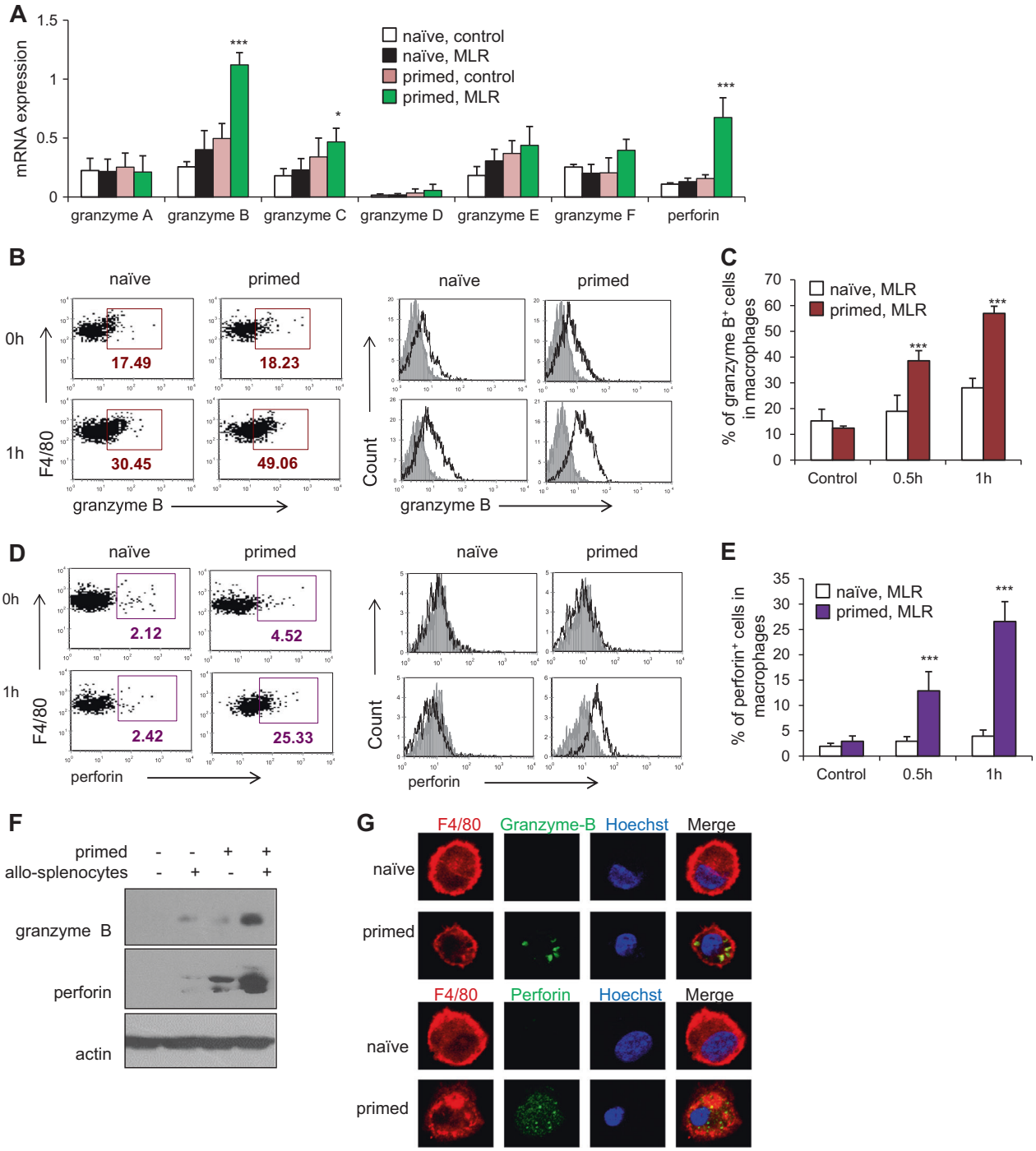

Fig. 4 Primed macrophages rechallenged with allo-antigens express more granzyme B and perforin ex vivo. Peritoneal macrophages were sorted from unimmunized B6 mice and from B6 mice that were immunized with BALB/c splenocytes for 10 days. **a** The expression of granzyme A–F and perforin in the sorted control and primed B6 $CD11b^+F4/80^+$ macrophages cocultured with or without the allogeneic donor BALB/c cells for 1 h were determined by real-time PCR. **b** Representative FACS staining is shown for granzyme B on $CD11b^+F4/80^+$ macrophages of the control and immunized B6 mice, and the macrophages were cultured with or without allogeneic BALB/c cells for 1 h. **c** The mean percentages of granzyme B^+ cells in $CD11b^+F4/80^+$ macrophages from the control and immunized B6 mice that were cultured with or without allogeneic cells for 1 h. **d** Representative FACS staining is shown for perforin on $F4/80^+CD11b^+$ macrophages of the control and immunized B6 mice. The macrophages were cultured with or without allogeneic BALB/c cells for 1 h. **e** The mean percentages of perforin$^+$ cells in $F4/80^+CD11b^+$ macrophages of the control and immunized B6 mice. The macrophages were cultured with or without allogeneic cells for 1 h. The expression of granzyme B and perforin in $F4/80^+$ macrophages of the control and immunized B6 mice (the macrophages were cultured with allogeneic donor BALB/c cells for 1 h) was observed using a western blot (**f**) and two-photon microscopy (**g**). One representative of three independent experiments with similar results is shown

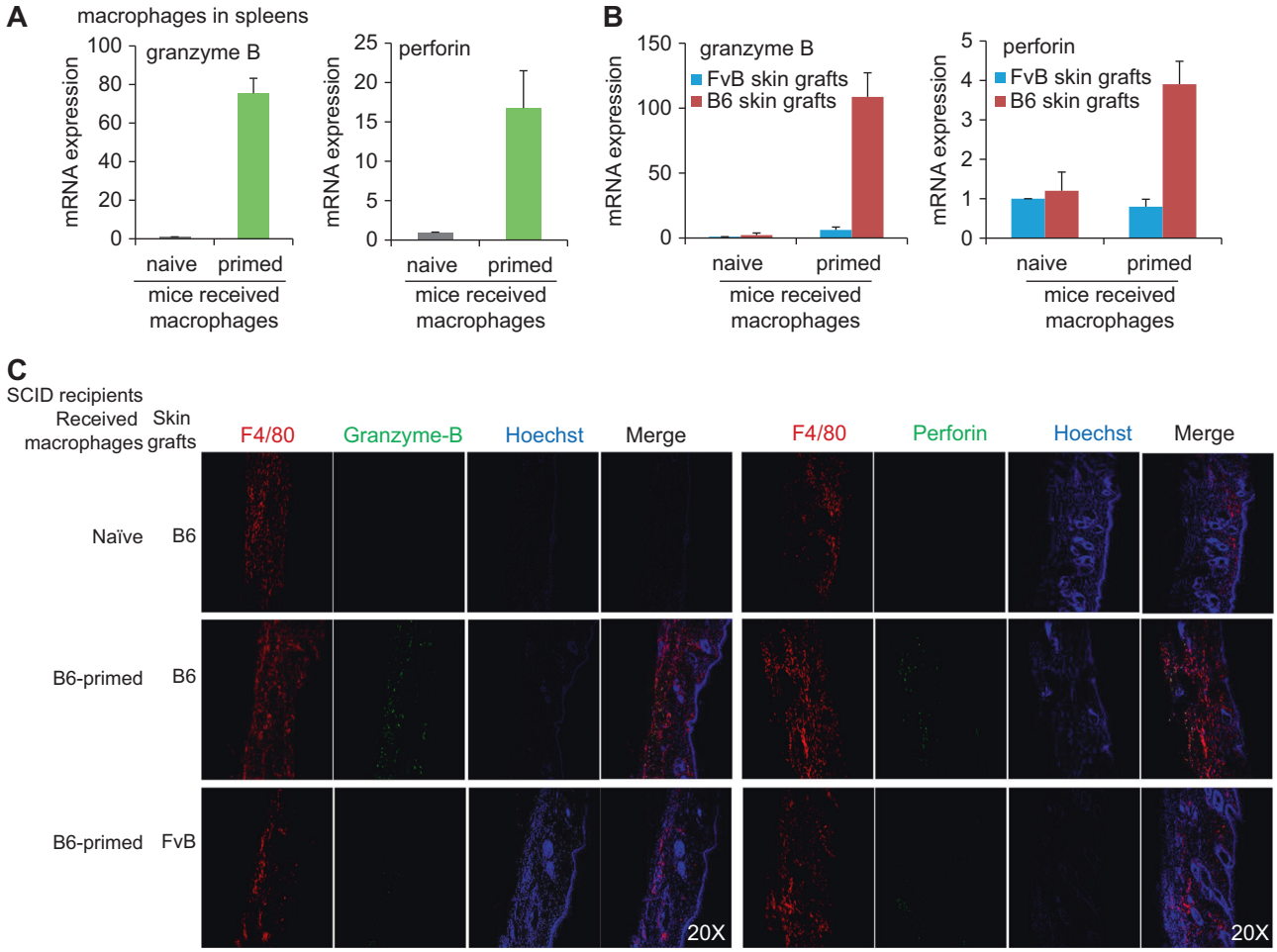

Fig. 5 Primed macrophages rechallenged with allo-antigens express more granzyme B and perforin in vivo. **a** The expression of granzyme B and perforin in sorted adoptive CD45.1$^+$ macrophages in the spleens of BALB/c skin-grafted Rag2 KO mice that received unimmunized and BALB/c cell-immunized CD45.1$^+$ macrophages was determined by real-time PCR. **b** The expression of granzyme B and perforin in the allogeneic B6 donor and in the 3rd party skin grafts in SCID mice that received unimmunized and B6 cell-immunized macrophages was determined by real-time PCR. **c** The expression of granzyme B and perforin was observed by fluorescence immunohistochemistry staining using a two-photon microscope. The skin samples were observed 10 days after grafting. The data are shown as the mean ± SD ($n = 3$), which are one representative of two independent experiments. ***$P < 0.001$ compared with the indicated groups

the third-party skin grafts. Although specific NK cell subpopulations against certain viruses have been discovered in recent years,[39] antigen-specific macrophage subsets and related receptors should be explored in detail.

Another important observation in the present study is that the primed macrophages in immunodeficient mice could efficiently proliferate upon re-encountering the allografts. Antigen-primed macrophages underwent a robust expansion in the immunodeficient mice that received allo-skin grafts, as indicated by the increased cell number and the high percentage of Ki67$^+$ cells in the adoptively transferred macrophages in SCID mice; in contrast, the unprimed macrophages failed to do so in the same model. More primed donor macrophages were detected in the SCID mice recipient tissues than those detected in SCID mice with naive macrophages after skin grafting. In addition, there were more primed macrophages infiltrated in the allogeneic skin grafts than there were in the third-party skin grafts. Thus, the primed macrophages significantly proliferated during their immune response to the allo-skin grafts.

Skin graft rejection is associated with a potent inflammatory immune response that involves both innate and adaptive immunity. Either CD4$^+$ or CD8$^+$ T cells activated through MHC-restricted recognition can lead to an acute rejection of the allograft.[40] B cells can act as antigen-presenting cells and can produce antibodies to mediate skin graft rejection.[41,42] NK cells are involved in allogeneic skin graft rejection via the direct killing of donor cells and through the production of pro-inflammatory cytokines, including IFN-γ and TNF-α.[43] A recent study showed that mast cell degranulation markedly accelerates skin rejection by enhancing neutrophil recruitment.[44] As important components of the innate immune system, macrophages can be polarized into pro-inflammatory M1 subsets, anti-inflammatory M2 subsets or other subsets in response to stimuli from the microenvironment,[45] and macrophages participate in allograft rejection via diverse mechanisms. First, macrophages can directly phagocytose allogeneic cells.[36] Second, M1 macrophages can secrete pro-inflammatory mediators, such as IL-1β, IL-2, IL-6, IL-12, IL-18, IFN-γ, TNF-α, and nitric oxide (iNOS), which activate and damage the microvasculature, recruit leukocytes, and induce donor-specific cytotoxic responses;[11,46] M2 macrophages can release anti-inflammatory IL-10 and TGF-β and can inhibit acute cellular rejection, but M2 macrophages contribute significantly to chronic rejection.[47,48] Finally, macrophages can also participate in rejection by modulating adaptive immunity. For example, IL-18 is

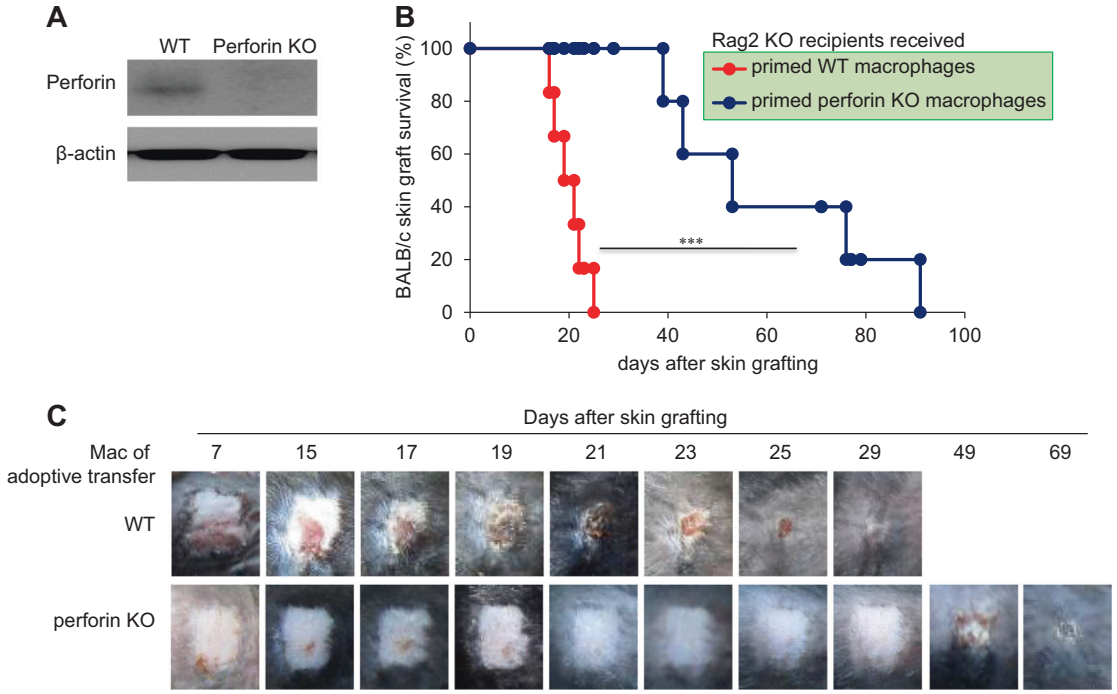

Fig. 6 Primed macrophages reject skin allografts partially via a perforin-dependent pathway. **a** Splenocytes from WT and perforin KO mice were stimulated with IL-2 (10 U/ml) for 4 h and were then immunoblotted with an anti-perforin antibody by western blot to confirm the deletion of perforin. **b** The survival curves of BALB/c skin allografts in Rag2 KO mice that received the sorted macrophages from BALB/c splenocyte-immunized WT or perforin KO mice before skin grafting are shown. ***$P < 0.01$ compared with the indicated groups. **c** Rag2 KO mice that received primed WT or perforin KO macrophages were transplanted with allogeneic donor BALB/c skin. The macroscopic pictures of allogeneic BALB/c skin grafts in Rag2 KO mice that received the sorted macrophages from BALB/c splenocyte-primed WT mice or perforin KO mice were taken at different time points

released by macrophages and is essential to establish adaptive Th1 responses (via IFN-γ production) against allo-skin grafts.[49] Regulatory macrophages can elicit Treg cells to control T cell immunity against allo-transplantation.[50] It is well known that the perforin pathway is the predominant effector in NK cell- and CD8+T cell-mediated organ and tumor rejection.[51,52] In the present study, we found that the perforin pathway was also employed by primed macrophages to reject allogeneic skin grafts. The adoptively transferred, primed macrophages that infiltrated the skin grafts expressed high levels of perforin and granzyme B. The wild-type primed macrophages rejected the skin grafts quickly, but the primed, perforin-deficient macrophages rejected the skin grafts in a significantly delayed manner. Thus, the primed macrophages rejected the skin grafts, at least partially, through the perforin pathway. The other pathways that are involved in the primed macrophage-mediated rejection of skin grafts require investigation. Nevertheless, these data also further support that the primed macrophages act as effector cells to directly reject allografts with the specificity of those from the adaptive immune system.

Overall, our findings show that the primed macrophages have the ability to reject allo-skin grafts with certain antigen specificity. Although the present study was performed in an allogeneic transplant mouse model and many issues remain to be addressed, the antigen-recognizing specificity of the primed macrophages, similar to NK cells,[39] may exist in mice.

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AUTHOR CONTRIBUTIONS

Z.C. designed and performed the adoptive transfer studies and analyzed the data; C. S. designed and performed the experiments with the cells and mice and analyzed the data; L.S. performed the real-time PCR assays and the molecular studies and analyzed the data; C.F. performed the immunostaining assays and flow cytometry assays; F.Y. designed and performed the ex vivo studies and analyzed the data; Y.X. performed histology assays; and Y.Z. provided overall supervision, designed the experiments, analyzed the data, and wrote the manuscript.

ADDITIONAL INFORMATION

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