

# Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells

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CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (Treg) are potent immunosuppressive cells active in controlling normal pathological immune responses. The mechanisms of this suppression have been investigated under various conditions. In this report, tumor necrosis factor-related apoptosis inducing ligand (TRAIL)/death receptor 5 (DR5) was explored as one of the pivotal factors for the suppression and cytotoxicity induced by CD4<sup>+</sup> CD25<sup>+</sup> Treg. Cell death was involved in the suppression induced by activated CD4<sup>+</sup> CD25<sup>+</sup> Treg *in vitro*. The induction of CD4<sup>+</sup> T cell death was not mediated by the CD95/CD95L pathway, but rather depended upon the upregulation of TRAIL in the Treg. Blocking the TRAIL/DR5 pathway resulted in a significant reduction of the suppressive activity as well as the cytotoxic effects of Treg *in vitro*. Activated Treg displayed TRAIL-dependent cytotoxicity against CD4<sup>+</sup> T cells *in vivo*. The prolonged survival of allogeneic skin grafts induced by Treg was inhibited by DR5-blocking antibodies. Our findings suggest that the TRAIL/DR5 pathway is one of the mechanisms used by Treg to regulate immune responses both *in vitro* and *in vivo*.

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Regulatory T cells (Treg) are believed to be responsible for inducing and maintaining peripheral immunologic tolerance.<sup>1,2</sup> Based upon their ontogeny and mode of action, several phenotypically distinct Treg populations have been identified, such as CD4<sup>+</sup> Treg, CD8<sup>+</sup> Treg and NKT cells. CD4<sup>+</sup> Treg is further divided into several subgroups, such as CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg, CD4<sup>+</sup> IL-10<sup>+</sup> Foxp3<sup>-</sup> T cells (Tr1), and CD4<sup>+</sup> TGF- $\beta$ <sup>+</sup> T cells (Th3).<sup>3,4</sup>

It has been demonstrated that the suppressive effects of Treg on effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells was via inhibition of IL-2 transcription, although other aspects of proximal signaling, such as CD69 upregulation, was often permitted.<sup>5–7</sup> Soluble factors IL-10 or TGF- $\beta$  were also implicated in the suppression by Treg via a cell contact-independent pathway.<sup>8,9</sup>

Several approaches were employed to molecularly characterize the mechanism underlying Treg-mediated cell-to-cell contact-dependent immune suppression. It was shown that triggering the glucocorticoid-induced TNF-receptor related protein (GITR), a marker constitutively expressed on Treg, diminished the cell-to-cell contact-dependent suppressive activity of Treg.<sup>10,11</sup> More recent studies addressed the function of CD80/CD86 ligands and cytotoxic T lymphocyte-associated antigen 4 (CTLA4), present on activated CD4<sup>+</sup> T cells. The absence of these molecules from effector T cells reduced the cell susceptibility to the suppression by CD4<sup>+</sup> CD25<sup>+</sup> Treg.<sup>12,13</sup> In addition, some members of Toll-like receptor (TLR), which constitutively expressed on Treg,

such as TLR 2 and TLR8, might participate in the Treg activation and expansion.<sup>14–16</sup>

Cytolytic activity was invoked as a possible mechanism of suppression by Treg. It was reported that activated human Treg expressed granzyme A and could kill T cells and antigen-presenting cells (APCs) through the perforin pathway *in vitro*.<sup>17</sup> However, by using granzyme B-deficient or perforin-deficient mice, a recent study showed that murine Treg-mediated immunosuppression occurred through a granzyme B-dependent but perforin-independent mechanism *in vitro* and that the death of effector T cells was a consequence of Treg.<sup>18</sup> It is unclear whether such phenomena exist *in vivo*.

These pioneering studies addressed possible mechanisms of immunosuppression by Treg under various conditions. Nevertheless, the molecular basis involved in Treg-mediated contact-dependent suppression *in vitro* or *in vivo* remains elusive to this date.

In the present study, we demonstrate that activated Treg suppress the proliferation and induced cell death of CD4<sup>+</sup> T cells. Most interestingly, such cell death was not mediated by the CD95/CD95L pathway, but was dependent upon TNF-related apoptosis inducing ligand (TRAIL), which might, in part, contribute to the antiproliferative effects of Treg. Blocking the TRAIL/death receptor 5 (DR5) pathway resulted in significantly reduced suppressive activity and cytotoxic effects of Treg *in vitro*. The cytotoxic effect of Treg on CD4<sup>+</sup> T cells was further explored *in vivo* and the prolonged survival of allogeneic skin grafts induced by Treg was found to

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**Abbreviations:** 7-AAD, 7-amino actinomycin D; acTregs, activated CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; DR5, death receptor 5; MACS, magnetic cell sorting; MHC, major histocompatibility complex; OVA, ovalbumin; PBMCs, peripheral blood monocytes; PI, propidium iodide; TCR, T-cell receptor; Tg, transgenic; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; WT, wild type

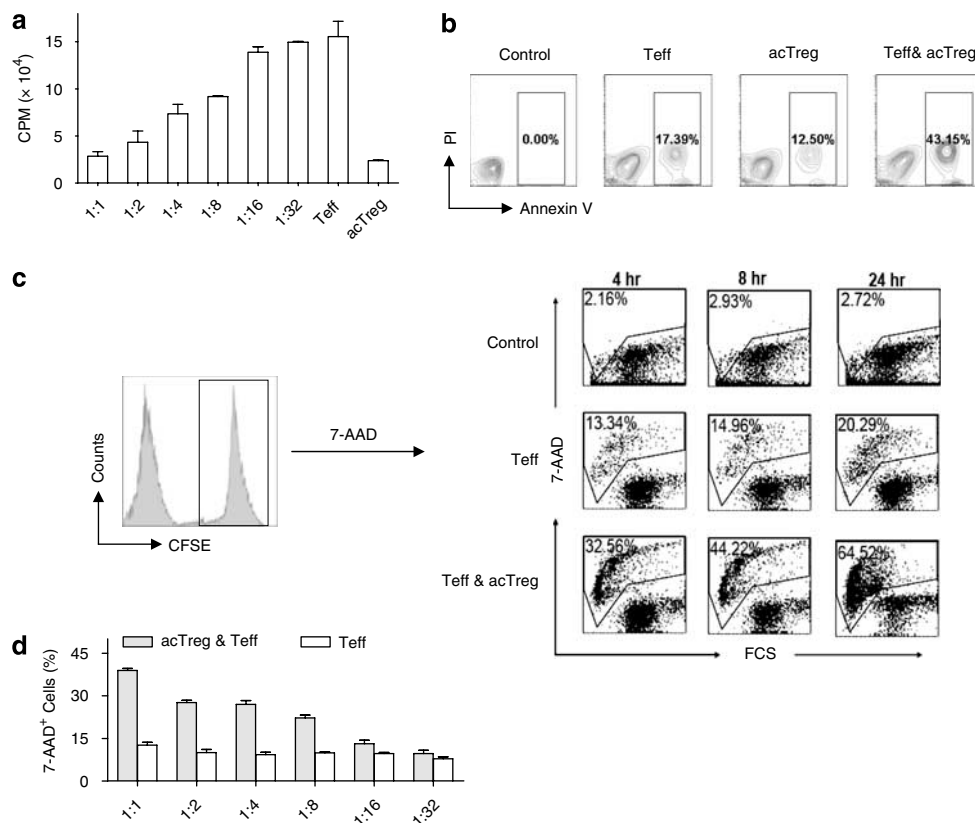
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be inhibited by DR5-blocking antibodies. These data suggest a possible role for TRAIL/DR5 in mediating cell contact-dependent T-cell suppression *in vitro* and *in vivo*.

## Results

**Activated Treg induce cell death and suppress proliferation of Teff cells.** To determine if CD4<sup>+</sup>CD25<sup>+</sup> Treg induced cell death during the suppression of CD4<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup> T cells were purified from naive mice (>95% Foxp3 positive). These naive CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg were found to inhibit the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells following polyclonal activation in a <sup>3</sup>H thymidine incorporation assay *in vitro* (Supplementary Figure 1a). Having been stimulated with plate-bound anti-CD3/CD28/high IL-2 for 5 days, the freshly purified CD4<sup>+</sup>CD25<sup>+</sup> Treg became enlarged and formed a pattern of open nuclear chromatin pattern observed under light microscopy (Supplementary Figure 1b) with the increased expression of CD69 and decreased expression of CD62L (Supplementary Figure 1c). Such activated CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg

(acTreg, anti-CD3/CD28/high IL-2 for 5 days) were subsequently co-cultured with Teff (CD4<sup>+</sup>CD25<sup>-</sup> T cells pre-stimulated with anti-CD3/CD28), which led to the suppression of Teff proliferation in a dose-dependent manner (Figure 1a). After staining such co-cultured cells (acTreg to Teff at a ratio of 1 : 1) with Annexin V and propidium iodide (PI), a 43.15% population of Annexin V<sup>+</sup>/PI<sup>-</sup> cells was observed, whereas the spontaneous apoptosis was 12.50% for acTreg and 17.39% for Teff (Figure 1b). By using an *in vitro* flow-based killing assay,<sup>19</sup> we further confirmed that acTreg had a cytotoxic effect on Teff which were time- (Figure 1c) and dose-dependent (Figure 1d). The percentage of 7-amino actinomycin D (7-AAD)-positive staining (a surrogate for cell death) of Teff in the co-culture was 32.56, 44.22 and 64.52% at 4, 8 and 24 h, respectively, whereas that of Teff alone without acTreg was 13.44, 14.96 and 20.29%, respectively, at corresponding time points. Very low background for the control was observed at all time points (Figure 1c). The death of Teff cells was induced specifically within Teff cells in co-culture (Supplementary Figure 2). This occurrence of apoptosis in the co-culture indicated that cell death might be involved in the suppression effect of acTreg.



**Figure 1** Activated Treg induce the death and suppressed the proliferation of Teff cells. (a) CD4<sup>+</sup>CD25<sup>+</sup> T cells (Treg) and CD4<sup>+</sup>CD25<sup>-</sup> T cells were obtained using MACS isolation. Treg were cultured in the presence of anti-CD3/CD28/high IL-2 (acTreg) for 5 days. CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured in the presence of anti-CD3/CD28 for 5 days and designated Teff. A volume of 1 × 10<sup>5</sup>/well Teff was cultured with the indicated numbers of acTreg. Teff alone and acTreg alone were taken as the control. <sup>3</sup>H-TdR incorporation was measured after cells were pulsed for 16 h during a total of 24 h of culture. (b) Teff, acTreg or mixture of Teff and acTreg at a 1 : 1 ratio were cultured for 8 h and the cells stained with Annexin V and PI. The control was the cells without Annexin V and PI staining. (c) CFSE-labeled Teff were subjected to the flow-based killing assay in which specific gating upon and analysis for 7-AAD incorporation was applied. CFSE-labeled Teff were cultured with acTreg or Teff at a 1 : 1 ratio for 4, 8 or 24 h and the percentage of 7-AAD-positive cells was determined. The control was cells with CFSE staining but without 7-AAD staining. (d) CFSE-labeled Teff (1 × 10<sup>5</sup>) were cultured with indicated numbers of acTreg or Teff for 8 h and the percentage of 7-AAD-positive cells was analyzed. Results are expressed as the mean of triplicate cultures and are representative of at least three experiments. \*P < 0.05, \*\*P < 0.01

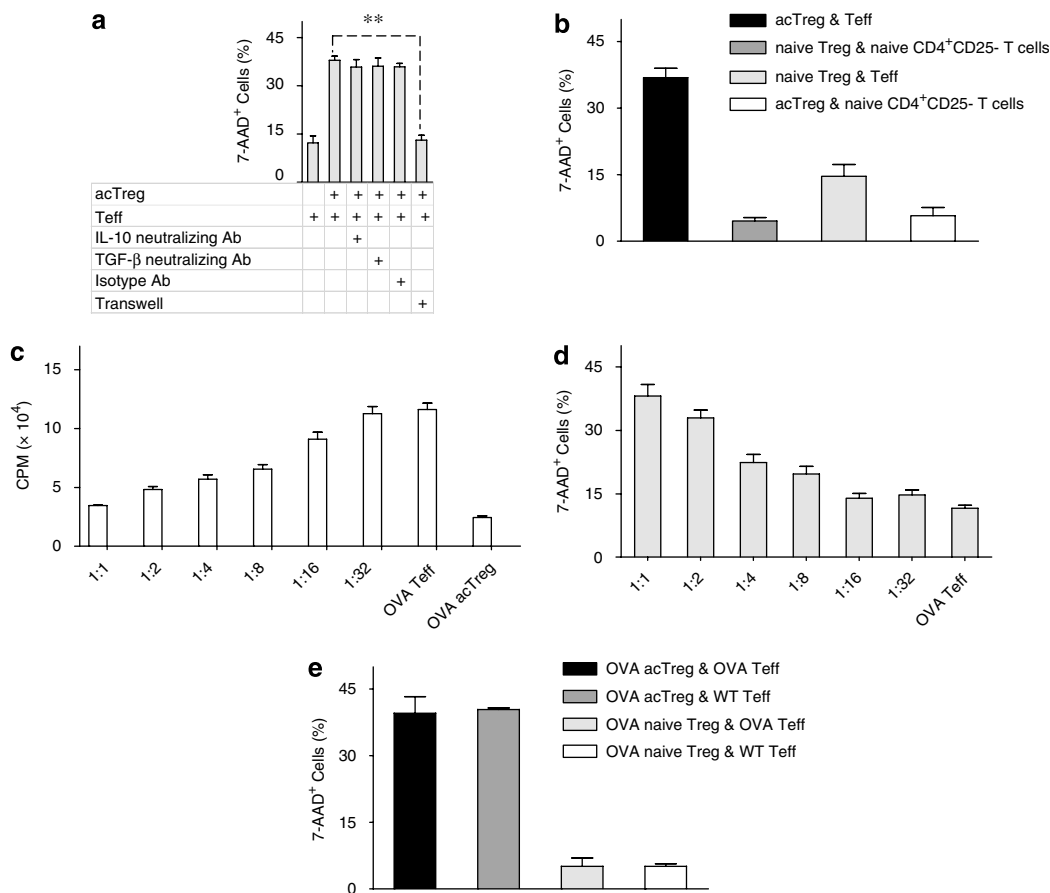
**Activated Treg have cytotoxic effects on Teff cells.** As it was shown that cell death and suppression occurred following in the co-culture of acTreg and Teff during the suppression, we next investigated possible contributing factors might be involved. Although a large amount of IL-10 and TGF- $\beta$  was released by the CD4<sup>+</sup>CD25<sup>+</sup> Treg during activation (Supplementary Figure 1d), these cytokines did not contribute to the cytotoxicity of acTreg. Neither IL-10 nor TGF- $\beta$  neutralizing antibody was found to significantly alter the cytotoxic effect mediated by acTreg as observed in Figure 2a ( $P > 0.05$ ). However, the cytotoxic effect of acTreg on Teff was abrogated when acTreg were separated from activated Teff by the semipermeable *trans*-well membrane (Figure 2a,  $P < 0.01$ ). These results indicated that the observed killing was cell contact-dependent.

In addition, no cytotoxic effect was found in the co-culture of either freshly isolated Treg and naive CD4<sup>+</sup>CD25<sup>-</sup> T cells, or acTreg and naive CD4<sup>+</sup>CD25<sup>-</sup> T. Freshly isolated Treg had a slight cytotoxic effect upon Teff pre-stimulated with anti-CD3/

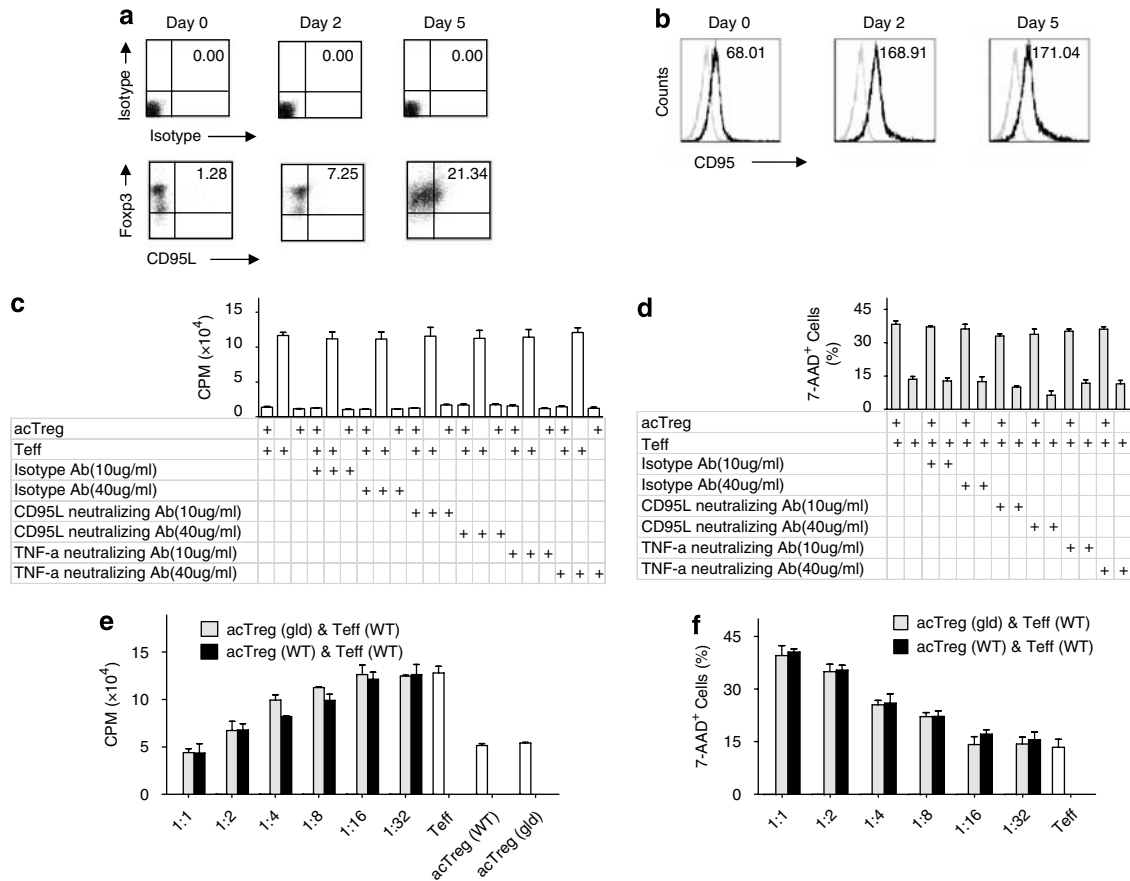
CD28; but a significant cytotoxic effect was observed in the co-culture of acTreg and Teff (Figure 2b).

To further investigate the killing effect of acTreg in antigen-specific immune responses besides anti-CD3/28 activation, we analyzed the function of acTreg in DO11.10 mice expressing transgenic  $\alpha\beta$  T-cell receptor (TCR) specific for the ovalbumin (OVA)<sub>323-339</sub> peptide.<sup>20</sup> The acTreg derived from TCR transgenic mice exerted similar suppressive and cytotoxic effects upon the activated Teff from TCR transgenic mice (Figure 2c and d). Furthermore, acTreg, but not naive Treg derived from TCR transgenic mice killed Teff from wild-type (WT) mice (Figure 2e), which implied that the cytotoxic effect of acTreg was not antigen-specific and a successful activation of Treg was crucial to its cytotoxic effect on Teff cells.

**The cytotoxic effect of acTreg on Teff cells is not mediated by CD95/CD95L.** The Fas/FasL pathway has been described as one of the main lytic mechanisms to



**Figure 2** Activated Treg have cytotoxic effects upon Teff cells. (a) AcTreg (anti-CD3/CD28/high IL-2 for 5 days) and Teff (CD4<sup>+</sup>CD25<sup>-</sup> T cells with anti-CD3/CD28 for 5 days) were separated by a semipermeable *trans*-well membrane and incubated for 8 h for the flow-based killing assay, and 7-AAD incorporation was measured. In some wells, the isotype antibody or neutralizing antibodies against IL-10 or TGF- $\beta$  were added. (b) Freshly isolated Treg or acTreg were cultured with freshly isolated naive CD4<sup>+</sup>CD25<sup>-</sup> T cells or Teff for 8 h for the flow-based killing assay and the percentage of 7-AAD-positive cells was analyzed. (c–e) Naive T cells isolated from splenocytes of DO11.10 Tg mice were stimulated with irradiated APCs and OVA<sub>323-339</sub> peptide with IL-2 (1000 U/ml for acTreg or 100 U/ml for Teff cells) for 5 days. A volume of  $1 \times 10^5$ /well Teff from Tg mice was cultured with the indicated numbers of acTreg from Tg mice (c–e) or WT mice (e). The 'OVA Teff' control was  $1 \times 10^5$ /well Teff alone in (c) and  $1 \times 10^5$ /well CFSE-labeled Teff added with the same numbers of Teff in (d). <sup>3</sup>H-TdR incorporation based proliferation assay (c) and Flow-based killing assay (d and e) were performed. Results are expressed as the mean of triplicate cultures and are representative of at least three experiments. \* $P < 0.05$ , \*\* $P < 0.01$



**Figure 3** The cytotoxic effects of acTreg are independent of the pathway of CD95/CD95L. (a) Treg were cultured in the presence of anti-CD3/CD28/IL-2 for 5 days. The expression of CD95L was detected at indicated time points using FACS. The fluorochrome-labeled secondary antibody controls were performed at each time points. (b) Naive CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured in the presence of anti-CD3/CD28 and the expression of CD95 was detected at the indicated time points using FACS. The fluorochrome-labeled secondary antibody controls were performed at each time points and overlapping in plots respectively. (c and d) acTreg (anti-CD3/CD28/high IL-2 for 5 days) and Teff (CD4<sup>+</sup>CD25<sup>-</sup> T anti-CD3/CD28 for 5 days) were mixed at 1:1 ratio and the proliferation assay (c) and flow-based killing assay (d) were performed as described above. CD95L-neutralizing antibody and isotype control were added at the beginning of the assays at indicated concentrations. (e and f) acTreg derived from *gld* mice or WT mice were cultured with activated Teff from WT mice at indicated ratios and the proliferation assay (e) and flow-based killing assay (f) were performed as described. Results are expressed as the mean of triplicate cultures and are representative of at least three experiments. \* $P < 0.05$ , \*\* $P < 0.01$

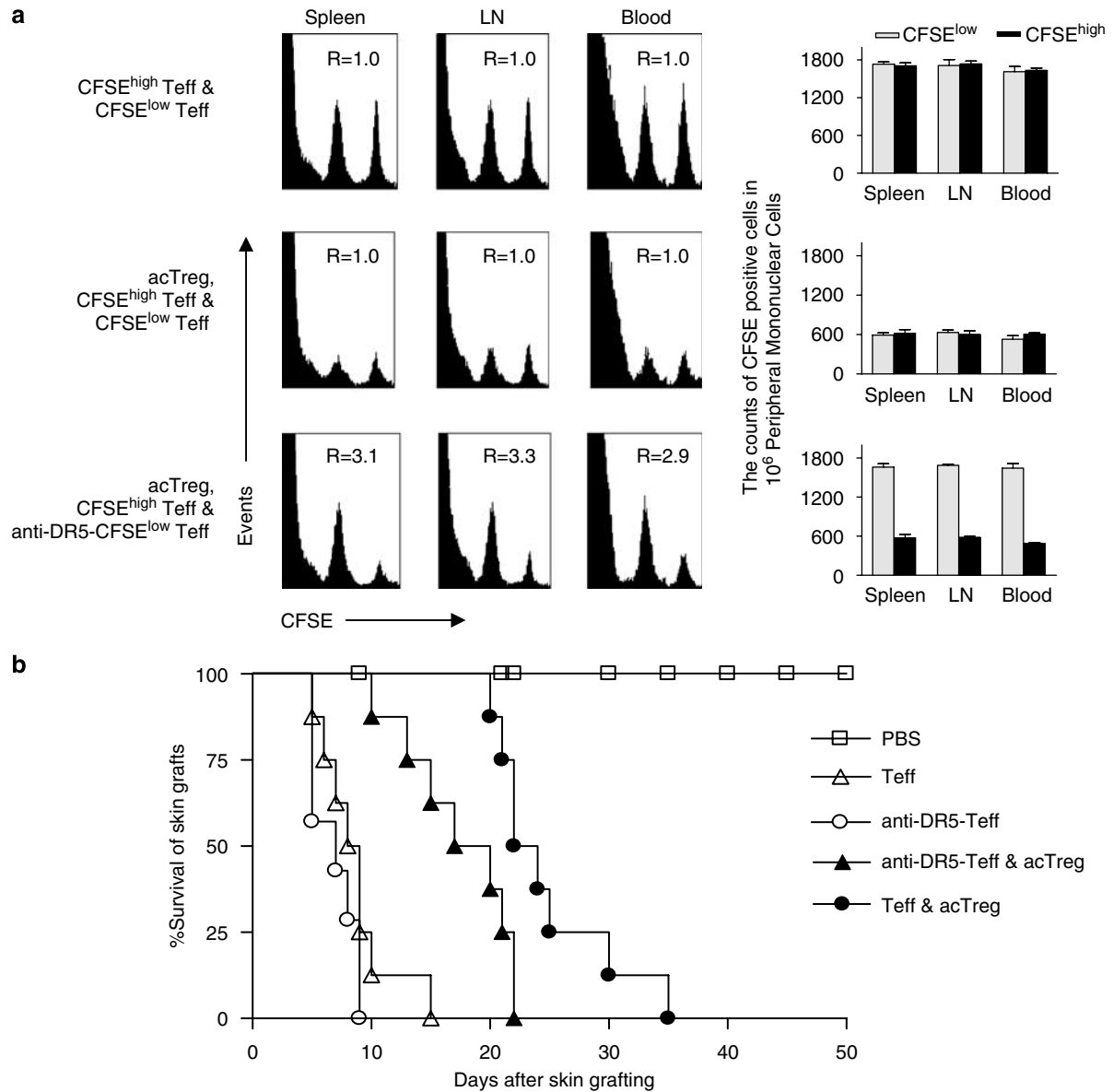
maintain T-lymphocyte homeostasis.<sup>21,22</sup> As the aforementioned studies revealed that Treg had a significant cytotoxic effect on Teff cells after activated, we investigated the CD95/CD95L pathway in the cytotoxic effect of acTreg on Teff. It was found that the expression of CD95L was upregulated following the activation of Treg (Figure 3a). The expression level of CD95 protein in Teff, as determined by mean fluorescence intensity (MFI), also increased after activation (Figure 3b). Although the expression levels of CD95/CD95L in acTreg and Teff were enhanced in activation, CD95L-neutralizing antibody failed to inhibit the suppressive or cytotoxic effect of acTreg on activated Teff cells (Figure 3c and d,  $P > 0.05$ ). Similarly, TNF- $\alpha$ -neutralizing antibody also did not influence such a suppressive or cytotoxic effect (Figure 3c and d,  $P > 0.05$ ). To further exclude the involvement of the CD95/CD95L pathway in the effect of Treg, Treg derived from *gld* mice (CD95L mutant) and those from WT mice were compared. There was no significant difference in either the suppression or cytotoxic effect between the acTreg from *gld* mice and those from WT mice (Figure 3e and f,  $P > 0.05$ ). These

results affirmed that the cytotoxic effect of acTreg on Teff cells was not mediated by CD95/CD95L.

**TRAIL/DR5 pathway is involved in the suppression and cytotoxic effects of acTreg on Teff cells.** Because neither CD95/CD95L nor TNF- $\alpha$ -neutralizing antibody was found to be involved in the suppressive or cytotoxic effect of acTreg, a possible role of the TRAIL/DR5 pathway was suspected. It was surprising to find that the acTreg, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells following anti-CD3/CD28/high IL-2 activation for 5 days, were nearly 95% TRAIL-positive by FACS analysis (Figure 4a). The experiment was repeated four times and a similar result was obtained. The expression level of DR5, as indicated by MFI, was greatly increased in the activated Teff (Figure 4b). The expression levels of TRAIL and DR5 appeared to correlate with the percentage of Teff cytotoxicity as induced by acTreg. The higher the expression of TRAIL/DR5, the greater the level of cytotoxicity was found (Figure 4c).

To further probe the role of TRAIL/DR5 in the effect of acTreg, DR5-blocking and TRAIL-neutralizing antibodies were applied in the subsequent assays. The suppressive





**Figure 5** AcTreg induce death of Teff *in vivo*. **(a)** Representative examples for *in vivo* killing of CFSE<sup>+</sup> targets. *nu/nu* mice were adoptively transferred with different combinations of cells as follows: a mixture of  $3 \times 10^7$  Teff previously labeled with a low CFSE fluorescence intensity ( $0.5 \mu\text{M}$ , CFSE<sup>low</sup>CD4) and  $3 \times 10^7$  Teff cells previously labeled with a high CFSE fluorescence intensity ( $5 \mu\text{M}$ , CFSE<sup>high</sup>CD4); a mixture of  $3 \times 10^7$  CFSE<sup>low</sup>Teff,  $3 \times 10^7$  CFSE<sup>high</sup>Teff and  $3 \times 10^6$  acTreg; a mixture of  $3 \times 10^7$  CFSE<sup>low</sup>CD4T cells pre-treated with DR5-blocking antibody,  $3 \times 10^7$  CFSE<sup>high</sup>CD4T cells and  $3 \times 10^6$  acTreg. Cells of each population were mixed in  $500 \mu\text{l}$  PBS for *i.v.* injection. Samples for submission to the *in vivo* cytotoxicity assay were acquired by collecting mononuclear cells from spleen, lymph nodes (LN) and blood 18 h after *i.v.* injection. Ratios were calculated as the percentages of CFSE<sup>low</sup>/CFSE<sup>high</sup> cells in a total of  $1 \times 10^6$  cells. The CFSE-positive target cells from *nu/nu* mice were determined using FACS. The ratio (R) between the amount of CFSE<sup>+</sup> and CFSE<sup>++</sup> cells was calculated. **(b)** BALB/c *nu/nu* mice were engrafted with tail skin from C57BL/6 mice and BALB/c mice, and inoculated with different combinations of cells as follows:  $3 \times 10^7$  Teff;  $3 \times 10^7$  Teff and  $3 \times 10^6$  acTreg;  $3 \times 10^7$  Teff pre-treated with DR5-blocking antibody;  $3 \times 10^6$  acTreg. Skin grafts were transplanted 1 day after cell transfer, and skin rejections were observed more than 100 days after the first grafts. Survival rates were analyzed using the Log-Rank test and all groups,  $n=8$

However, the ratio of CFSE<sup>low</sup> to CFSE<sup>high</sup> was kept at 1.0. When  $3 \times 10^7$  CFSE<sup>high</sup> and  $3 \times 10^6$  acTreg were adoptively transferred with  $3 \times 10^7$  CFSE<sup>low</sup> Teff pretreated with DR5-blocking antibody, more interestingly, CFSE<sup>high</sup> Teff decreased more rapidly than those of CFSE<sup>low</sup>. The ratios of CFSE<sup>low</sup> to CFSE<sup>high</sup> reached around 3 (Figure 5a), and similar results were found in peripheral blood at 4, 12 and 24 h post-transfer (Supplementary Figure 4a). Collectively,

these data indicated that acTreg accelerated the apoptosis of Teff *in vivo* and such cytotoxicity was inhibited by DR5-blocking antibody.

In further experiments, two contiguous skin grafts from C57BL/6 and BALB/c mice were applied to the back of *nu/nu* BALB/c mice that previously received either syngeneic acTreg or Teff. Syngeneic skin (from BALB/c mice) survived indefinitely in all groups. Allogeneic grafts (from C57BL/6

mice) survived over 100 days in phosphate-buffered saline (PBS)-injected *nu/nu* BALB/c mice, whereas allogeneic grafts were rejected by *nu/nu* BALB/c mice that received syngeneic  $3 \times 10^7$  Teff from BALB/c mice (Figure 5b). *Nu/nu* BALB/c mice that had received syngeneic  $3 \times 10^6$  acTreg and  $3 \times 10^7$  Teff from BALB/c mice rejected allogeneic grafts slowly. Importantly, allogeneic grafts were rejected more quickly by *nu/nu* BALB/c mice that received syngeneic  $3 \times 10^6$  acTreg and  $3 \times 10^7$  anti-DR5 pretreated Teff from BALB/c mice as compared to those receiving syngeneic  $3 \times 10^6$  acTreg and  $3 \times 10^7$  Teff (Figure 5b). Repetitive injection of DR5-blocking antibodies slightly decreased the survival rate (Supplementary Figure 4b). Taken together, these data demonstrated that Treg prolonged the survival of skin grafts *in vivo*, a response that was inhibited by DR5-blocking antibody.

## Discussion

We demonstrate that TRAIL/DR5 is one of the pivotal elements in the cytolytic activity-mediated suppression of Treg *in vitro* and *in vivo*. This TRAIL/DR5-mediated function of Treg was detected in the suppression assays with pre-activated Treg and effector T cells, or with Treg and naïve effector T cells in the presence of anti-CD3/28 (Supplementary Figure 5). TRAIL/DR5 was involved in activated Treg-induced Teff death in skin grafts model. Furthermore, Treg activated *in vivo* did express TRAIL (Supplementary Figure 6). The evidences support that TRAIL/DR5 is one of the mechanisms to control the magnitude of Treg-mediated suppression.

It was generally regarded that TRAIL selectively induced apoptosis in tumorigenic or transformed cells, but not in most normal cells.<sup>25</sup> However, Zhang *et al.*<sup>26</sup> found that Th1 cells were sensitive to TRAIL-mediated apoptosis and that blocking of TRAIL-mediated apoptosis promoted Th1 differentiation. A similar effect was also observed in our study within Teff cells. Mice deficient in TRAIL had a severe defect in thymocyte apoptosis and were also hypersensitive to collagen-induced arthritis and streptozotocin-induced diabetes.<sup>27</sup> It is intriguing to explore if dysfunction of Treg contributes to the defect of TRAIL-deficient mice.

Various molecular and cellular events have been described to explain the mechanisms of Treg-mediated suppression. However, so far none of the proposed mechanisms can explain all aspects of the suppression. It is probable that various combinations of several mechanisms are operating, depending on the milieu and the type of immune responses, although there might be a single key mechanism that has a predominant role.<sup>28</sup> In addition to the illustration of IL-2 for initiation of the function of Treg,<sup>29</sup> GITR ligands may be involved in conferring resistance to suppression by  $CD4^+CD25^+$  Treg by making effector T cells less susceptible.<sup>30</sup> Gondek *et al.*<sup>18</sup> showed that the cell-dependent killing of GITR by Treg appeared to involve GrzB killing of the target cells. We also found GITR-blocking antibody decreased the death of Teff induced by Treg, but no synergistic effect of GITR- and DR5-blocking antibodies was found (Supplementary Figure 7). It is possible that the cell death for GITR group or DR5 group already approached to that of Teff alone so that

no additional blocking of death could be observed in the combination antibody group.

We and three other groups<sup>17,18,31</sup> reported that Treg had cytotoxic effects on effector cells *in vitro*. Human Treg activated by anti-CD3/CD46 displayed perforin-dependent cytotoxicity against autologous target cells *in vitro*.<sup>17</sup> The perforin/granzyme pathway was also considered to be one of the mechanisms by which murine Treg killed B lymphocytes.<sup>31</sup> However, Gondek *et al.* showed that the suppression by  $CD4^+CD25^+$  Treg activated by CD3 involved a granzyme B-dependent, perforin-independent mechanism *in vitro*.<sup>18</sup> We observed that in the suppression assay, there was a minor, yet significant distinction ( $P=0.0425$ , Supplementary Figure 8b) between Treg derived from perforin<sup>-/-</sup> mice and those from WT mice; and in the killing assay, that distinction was slight but significant as well ( $P=0.0420$ , Supplementary Figure 8c). The data from the TRAIL/DR5-blocking assay were much more impressive as compared to those of the perforin and CD95 pathways. Our results revealed that TRAIL/DR5, but not CD95/CD95L, played a pivotal role in the cytotoxic effect of Treg during the suppression process.

Suppressive mechanisms of Treg *in vivo* have not been well defined. The majority of models provide strong evidence for dependence on IL-10 or TGF- $\beta$  leading to bystander suppression.<sup>8,23,32</sup> It was shown that IL-10 and CTLA-4 were required for abrogated rejection of skin grafts mediated by naive  $CD45RB^{\text{high}}$   $CD4^+$  T cells of Treg *in vivo*.<sup>33</sup> There were more questions raised than answered concerning the mode of action and immunological regulatory molecular mechanisms mediated by Treg *in vivo*. Thus, we investigated how Treg exerted effects upon  $CD4^+$  T cells *in vivo*. We showed that Treg were capable of killing  $CD4^+$  T cells *in vivo* and that such cytotoxicity were inhibited by DR5-blocking antibody. We found that Treg prolonged the survival of skin grafts *in vivo*, an event that was inhibited by DR5-blocking antibody.

In addition to  $CD4^+$  T cells, the cytotoxic effect of activated Treg on  $CD8^+$  T cells,<sup>17</sup> immature DC and mature DC<sup>17</sup> and recently reported B cells<sup>31</sup> were defined by us and other groups. We found that Treg were capable of killing activated  $CD4^+$  T cells,  $CD8^+$  T cells and DCs (data not shown). Apoptosis in the immune system is critical for maintaining self-tolerance and preventing autoimmunity. The death of  $CD4^+$  T cells,  $CD8^+$  T cells and even DC,<sup>34</sup> all contribute to self-tolerance. Our findings and other groups' work revealed that the suppression mediated by Treg appears, at least in part, to be by the induction of apoptosis in these effector cells.

In our study, it appeared that TRAIL was important in the suppression mediated by Treg via apoptosis induction *in vitro* and *in vivo*. Treg are one of the pivotal components in maintaining immune homeostasis and controlling peripheral tolerance. Our findings may reveal a novel pathway by which TRAIL/DR5 regulates the immune responses.

## Materials and Methods

**Animals.** BALB/c (H-2<sup>d</sup>) mice, C57BL/6 (H-2<sup>b</sup>) mice and BALB/c (H-2<sup>d</sup>) *nu/nu* mice were purchased from the Experimental Animal Center of the Chinese Academy of Science, Shanghai. DO11.10 mice expressing a TCR specific for H-2<sup>d</sup>/OVA<sub>323–339</sub>, *gld* mice carrying a null-function mutation of the CD95L gene (B6Smn.C3HFasLgld, H-2<sup>b</sup>) were originally obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the Experimental Animal Center of the

Chinese Academy of Science, Shanghai. Mice (6–8 weeks old) were kept in a specific pathogen-free facility at the Experimental Animal Center of the Chinese Academy of Science. Animal care and use were carried out in compliance with institutional guidelines.

**T-cell isolation and stimulation.** CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from murine splenocytes by magnetic separation with magnetic cell sorting (MACS) (Miltenyi Biotech, Germany) according to manufacturer's instructions. The purities of CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells were determined at 95–97% by FACS analysis. Naive CD4<sup>+</sup>CD25<sup>+</sup> T cells (Treg) isolated from splenocytes of BALB/c, C57BL/6 or *gld*/C57BL6 mice were stimulated with plate-bound anti-CD3 (30 μg/ml), anti-CD28 (15 μg/ml) and IL-2 (1000 U/ml) (Chiron, USA) in complete RPMI1640 (GIBCO) medium (RPMI medium 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine/100 U/ml, and penicillin/100 μg/ml streptomycin/0.05 M 2-mercaptoethanol) for 5 days. Naive CD4<sup>+</sup>CD25<sup>-</sup> T cells were stimulated with plate-bound anti-CD3 (10 μg/ml), anti-CD28 (5 μg/ml) and IL-2 (100 U/ml) in complete RPMI1640 medium for 5 days. Alternatively, naive T cells (1 × 10<sup>6</sup>) isolated from splenocytes of DO11.10 transgenic (Tg) mice were stimulated with 4 × 10<sup>6</sup> irradiated APCs from BALB/c mice, 0.6 μM OVA<sub>323–339</sub> peptide (OVA) (GL Biochem, Shanghai, China) and IL-2 (1000 U/ml for CD4<sup>+</sup>CD25<sup>+</sup> T cells or 100 U/ml for CD4<sup>+</sup>CD25<sup>-</sup> T cells) in complete RPMI1640 for 5 days. Such activated Treg were defined as acTreg and activated CD4<sup>+</sup> T cells were applied as effector T cells and designated as Teff. AcTreg and Teff were used in subsequent experiments.

**Flow cytometry.** All mAb were obtained from eBioscience except for functional grade purified anti-IL-10-, anti-TGF-β- and anti-DR5-blocking antibodies from R&D. Unless otherwise specified, purified cells were stained with directly conjugated antibodies: anti-CD4-FITC, anti-CD69-FITC, anti-CD62L-FITC, anti-DR5-PE or stained with indirectly conjugated antibodies: anti-CD95, anti-CD95L, anti-TRAIL. The fluorochrome-labeled isotype control antibodies or fluorochrome-labeled secondary antibodies served as controls. For CD4 and CD62L, the control were FITC-rat IgG2b and FITC-rat IgG2a isotype control, respectively, for CD69 and DR5 were FITC-Armenian hamster IgG isotype control. For CD95, CD95L and TRAIL, the controls were FITC-anti-mouse IgG, FITC anti-Armenian hamster IgG and FITC anti-rat IgG, respectively. For intracellular Foxp3 staining, cells were fixed and permeabilized using reagents provided with the anti-Foxp3-PE-Cy5 (FJK-16s) antibody. All samples also included staining with the appropriate isotype control (IgG2a-PE-Cy5 (eBR2a)). The cells were analyzed on a FACSCalibur flow cytometry.

**Proliferation assay.** Purified naive CD4<sup>+</sup>CD25<sup>-</sup> T cells alone or co-cultured with naive CD4<sup>+</sup>CD25<sup>+</sup> T cells (Treg) of the indicated number in 96-well plates were incubated in the presence or absence of soluble anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) for 96 h. For the suppression assay of acTreg, pre-activated Teff alone (Teff control) or co-cultured with acTreg (as described above) of the indicated number were incubated in 96-well plates for 24 h in complete RPMI medium containing 100 U/ml IL-2. In some experiments, CD95L, TRAIL-neutralizing antibody or DR5-blocking antibody was added at indicated concentrations at the beginning of the culture. <sup>3</sup>H-TdR was added during the last 16 h of culture. All experiments were performed in triplicate.

**In vitro cytotoxicity assay.** A flow-based killing assay was developed to measure *in vitro* cellular cytotoxicity in the manner described in Lecoeur *et al.*<sup>19</sup> Naive or activated Teff cells were washed with PBS, resuspended at 1 × 10<sup>6</sup> cell/ml, and then labeled at 37°C for 15 min with 200 nM final concentration of CFSE (Sigma). Labeling reactions were stopped with complete RPMI medium. Labeled cells (1 × 10<sup>5</sup>) were added to 96-well V-bottom tissue culture-treated plates (Corning Costar) along with Treg or acTreg of the indicated number in complete RPMI medium containing 100 U/ml IL-2 for all time points shown. The Teff control was 1 × 10<sup>5</sup> labeled Teff cells co-cultured with 1 × 10<sup>5</sup> Teff. Immediately before analysis, 1 μg/ml (final concentration) 7-AAD (Sigma) was added to each sample. 7-AAD incorporation was used as a surrogate marker for late cell death/apoptosis, intercalating with DNA in cells that have lost membrane integrity. In some experiments, CD95L, TRAIL-neutralizing antibody or DR5-blocking antibody was added to the co-culture at indicated concentrations. 2 or 20 μM portion of caspase inhibitor z-VAD-fmk (CalBiochem) was added to inhibit caspase activity.

**In vivo cytotoxicity assay.** An *in vivo* killing assay was developed to measure *in vivo* evaluation of cytotoxic activity in the manner described by Chen *et al.*<sup>23</sup> and Nelson *et al.*<sup>24</sup> Activated Teff from BALB/c mice were labeled with either 0.5 or 5 μM CFSE (namely, CFSE<sup>low</sup> or CFSE<sup>high</sup>). For *i.v.* injection, 3 × 10<sup>7</sup> cells of each population with or without 3 × 10<sup>6</sup> acTreg were mixed in 200 ml of PBS per recipient mouse (BALB/c *nu/nu* mice). In some experiments, the CFSE<sup>low</sup> population was pretreated with anti-DR5-blocking antibody. *In vivo* cytotoxicity was determined by collecting mononuclear cells from spleen, lymph nodes and blood from recipient mice 18 h after *i.v.* injection. Fluorescent target cell populations were detected by flow cytometry.

**Western blot analysis.** AcTreg and Teff were generated as described above. In some experiments, Treg and/or Teff were also cultured with 10 μg/ml DR5-blocking antibody. The cells were lysed in lysis buffer (50 mM HEPES, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1 × protease inhibitor cocktail from Roche) and cell lysates (50 μg) were subjected to Western blot analysis by probing with a rabbit monoclonal antibody made against the cleavage fragment of caspase 3 (Asp175, Cell signaling). The blots were then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies and developed using the chemiluminescent method (Pierce). The membranes were also probed with anti-GAPDH (R&D) to ensure equal loading.

**Skin graft preparation.** Activated Teff cells (3 × 10<sup>7</sup>) with or without anti-DR5-blocking antibody treatment were mixed with acTreg (3 × 10<sup>6</sup>) and adoptively transferred *i.v.* into recipients (BALB/c *nu/nu* mice). Allogeneic skin was grafted 1 day after cell transfer. The survival of the grafts was followed for more than 100 days. Skin grafts were prepared and transplanted as previously described by Ref.<sup>35</sup> Briefly, full-thickness tail skin (0.8 × 0.8 cm) was grafted onto the lateral flank of recipient mice. A suture was sewn onto each corner of the graft and subsequently bandaged. Bandages were removed 1-week post-transplantation. Grafts were monitored every other day and scored as rejected when >75% or more of the original graft tissue had been lost or became necrotic as assessed by visual examination.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)