Identification of $CD3^+CD4^-CD8^-$ T Cells as Potential Regulatory Cells in an Experimental Murine Model of Graft-Versus-Host Skin Disease (GVHD)

Fumi Miyagawa[1], Naoko Okiyama[1], Vadim Villarroel[1] and Stephen I. Katz[1]

We have developed K14-mOVA transgenic (Tg) mice that express membrane-associated ovalbumin (mOVA) under the control of a K14 promoter, as well as double Tg mice, by crossing them with OT-I mice that have a TCR recognizing the OVA peptide. When injected with $CD8^+$ OT-I cells, K14-mOVA Tg mice develop graft-versus-host disease (GVHD), whereas double Tg mice are protected. This suggests that, in double Tg mice, regulatory mechanisms may prevent infused OT-I cells from inducing GVHD. We demonstrated that, after adoptive transfer, $TCR\alpha\beta^+CD3^+CD4^-CD8^-NK1.1^-$ double-negative (DN) T cells are increased in the peripheral lymphoid organs and skin of double Tg mice and exhibit a $V\alpha2^+V\beta5^+$ TCR that has the same TCR specificity as OT-I cells. These DN T cells isolated from tolerant double Tg mice proliferated in response to OVA peptide and produced IFN-γ in the presence of IL-2. These cells could also suppress the proliferation of OT-I cells and were able to specifically kill activated OT-I cells through Fas/Fas ligand interaction. These findings suggest that DN T cells that accumulate in double Tg mice have regulatory functions and may have a role in the maintenance of peripheral tolerance *in vivo*.

Journal of Investigative Dermatology (2013) **133,** 2538–2545; doi:10.1038/jid.2013.212; published online 11 July 2013

INTRODUCTION

Multiple mechanisms of immune tolerance to self-antigen are required to prevent autoimmunity. Some self-reactive T cells are deleted during thymic differentiation (Kappler *et al.*, 1987; Kisielow *et al.*, 1988; Pircher *et al.*, 1989), but others escape clonal deletion and go on to populate the periphery (Bouneaud *et al.*, 2000; Walker and Abbas, 2002). These self-reactive T cells are controlled by peripheral tolerance mechanisms (Jones *et al.*, 1990; Rocha and von Boehmer, 1991) acting either directly on the self-reactive T cell (ignorance, anergy, phenotype skewing, apoptosis) or indirectly via additional cells (tolerogenic dendritic cells, regulatory T cells (Tregs)) (Walker and Abbas, 2002). There is growing evidence that these regulatory cells have important roles in the maintenance of immune tolerance to self-antigens and foreign antigens. A variety of Tregs have been described, including $CD4^+$ (Gonzalez *et al.*, 2001; Sakaguchi *et al.*, 2008; Shevach, 2011), $CD8^+$ (Jiang and Chess, 2000), $CD4^-CD8^-$ double-negative (DN) $TCR\alpha\beta^+$ cells (Zhang *et al.*, 2000; Priatel *et al.*, 2001), $\gamma\delta TCR^+$ cells (Hayday and Tigelaar, 2003), and natural killer T cells (Sharif *et al.*, 2002; Duarte *et al.*, 2004). To date, $CD4^+CD25^+$ T cells are the most extensively studied Tregs that have an important role in preventing the development of autoimmune diseases and allograft rejection (Sakaguchi *et al.*, 2008; Shevach, 2011).

A novel subset of antigen (Ag)-specific $TCR\alpha\beta^+CD3^+CD4^-CD8^-NK1.1^-$ DN regulatory T cell has been identified (Zhang *et al.*, 2000) and has shown to be able to specifically suppress the activity of auto-, allo-, or xeno-reactive $CD8^+$ T cells (Zhang *et al.*, 2000; Chen *et al.*, 2003; Ford *et al.*, 2007), $CD4^+$ T cells (Chen *et al.*, 2003, 2005; Zhang *et al.*, 2007; Voelkl *et al.*, 2011), B cells (Hillhouse *et al.*, 2010; Zhang *et al.*, 2007), or dendritic cells (Gao *et al.*, 2011) in several animal models and humans. Unlike $CD4^+CD25^+$ T cells that execute their function in an Ag-nonspecific manner (Sakaguchi *et al.*, 2008; Shevach, 2011), DN Tregs can downregulate immune responses in an Ag-specific manner both *in vitro* and *in vivo* (Zhang *et al.*, 2000; Ford *et al.*, 2002; Zhang *et al.*, 2002; Chen *et al.*, 2003). This population of DN Tregs differs from other Tregs in its surface marker expression, cytokine profile, and mechanism of suppression, and can specifically suppress $CD8^+$ and $CD4^+$ T cells that are primed against the same alloantigen (Zhang *et al.*, 2000; Ford *et al.*, 2002; Young and Zhang, 2002; Chen *et al.*, 2003).

We developed transgenic (Tg) mice that express ovalbumin (OVA) under the control of the keratin 14 promoter (K14-mOVA). After passive transfer of OT-I cells, the $CD8^+$ T cells transfected with a TCR that recognizes OVA peptide in

[1]*Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA*

Correspondence: Stephen I. Katz, Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA. E-mail: katzs@od.niams.nih.gov

Abbreviations: Ag, antigen; CFSE, carboxyfluorescein succinimidyl ester; DN, double negative; FasL, Fas ligand; GFP, green fluorescent protein; GVHD, graft-versus-host disease; K14, keratin 14; LN, lymph node; OVA, chicken ovalbumin; SDLN, skin-draining lymph node; Tg, transgenic; Treg, regulatory T cell

Received 13 September 2012; revised 25 March 2013; accepted 2 April 2013; accepted article preview online 6 May 2013; published online 11 July 2013

association with major histocompatibility complex class I, these mice develop graft-versus-host disease (GVHD)–like disease including skin lesions and weight loss (Shibaki *et al.*, 2004; Miyagawa *et al.*, 2008). However, double Tg mice that have both K14-mOVA and OT-I TCR (Vα2 and Vβ5) transgenes do not develop GVHD after transfer of OT-I cells, suggesting that peripheral tolerance may be involved.

In this study, we demonstrate that the number of $V\alpha2^+V\beta5^+CD3^+CD4^-CD8^-$ DN T cells is significantly increased in peripheral lymphoid organs, as well as in the skin of double Tg mice. We further demonstrate that the DN T cells from the peripheral lymphoid organs of double Tg mice are able to specifically suppress and kill OT-I cells, $CD8^+$ T cells carrying the same TCR specificity. Thus, these DN T cells are able to suppress and kill autoreactive $CD8^+$ T cells in this model and may participate in the immunologic tolerance exhibited by the double Tg mice when OT-I cells are adoptively transferred.

RESULTS

Double Tg mice do not develop GVHD after transfer of OT-I cells

When injected with OT-I cells, K14-mOVA Tg mice regularly develop GVHD-like disease, including weight loss and inflammatory skin lesions with histological changes, whereas double Tg mice generated by crossing K14-mOVA Tg mice with OT-I mice do not develop GVHD (Figure 1a–c). When we transferred green fluorescent protein $(GFP)^+$ OT-I cells to these double Tg mice, GFP^+ OT-I cells did not proliferate in the skin-draining lymph nodes (SDLNs) or spleens of double Tg mice compared with those transferred to K14-mOVA mice (Figure 1d and e). Although the adoptively transferred GFP^+ OT-I cells migrated to the ear skin of K14-mOVA Tg mice, the percentages of GFP^+ OT-I cells in the skin of double Tg mice were significantly decreased compared with those transferred to K14-mOVA Tg mice (Figure 1f). Flow cytometric analysis demonstrated that the adoptively transferred GFP^+ OT-I cells did not show an activated phenotype in the SDLNs of double Tg mice (Okiyama *et al.*, unpublished data). These results suggest that regulatory mechanisms prevent these OT-I cells from inducing GVHD in double Tg mice. Even adoptive transfer of $CD44^{high}CD62L^{low}$ OT-I cells activated *in vitro* with OVA peptides and IL-2 that cannot migrate to LNs did not cause GVHD in the double Tg mice (Supplementary Figure S1 online).

$CD3^+CD4^-CD8^-$ cells (DN T cells) expressing $V\alpha2^+V\beta5^+$ TCR are significantly increased in double Tg mice

When percentages of $CD4^+$, $CD8^+$, and DN cells were compared in SDLNs of C57BL/6, K14-mOVA, and double Tg

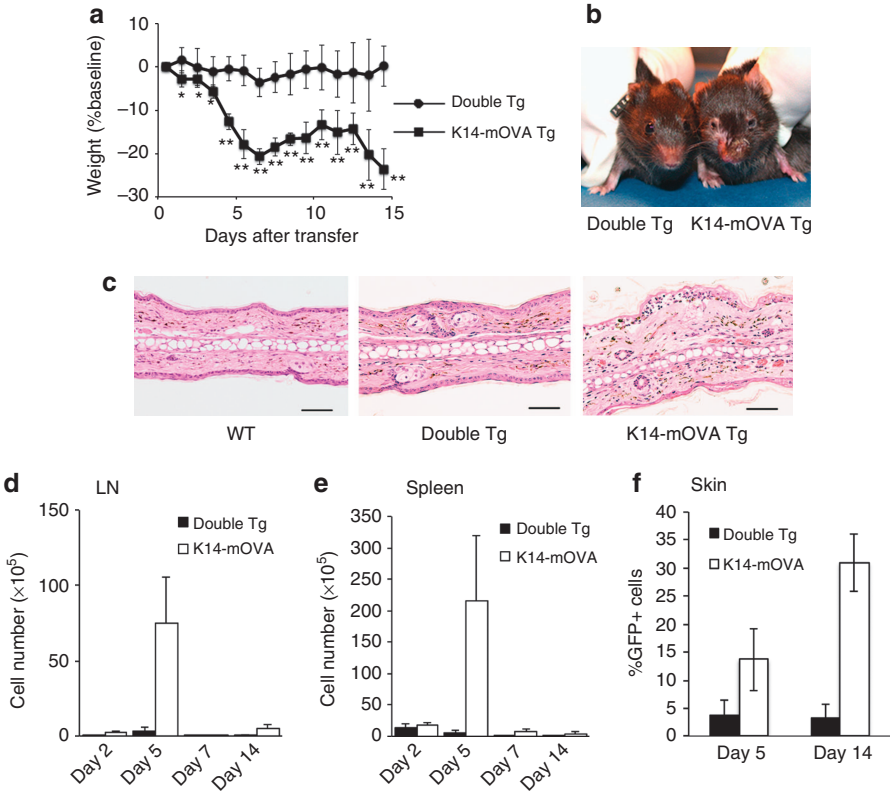

Figure 1. Double transgenic (Tg) mice do not develop graft-versus-host disease (GVHD). (**a**) Weight-course graph. Five million OT-I cells were adoptively transferred into K14-mOVA and double Tg mice. The mice were weighed daily for 2 weeks. (**b**) Clinical photographs and (**c**) hematoxylin and eosin (H&E)–stained ear tissues of mice 14 days after transfer of 5×10^6 OT-I cells. $^*P<0.05$ and $^{**}P<0.01$. Scale bars = 50 μm. (**d**, **e**) The numbers of GFP^+ OT-I cells in the (**d**) SDLNs (cervical LNs, inguinal LNs, and axillary LNs) and (**e**) spleens of three mice each at 2, 5, 7, and 14 days after adoptive transfer of 5×10^6 GFP^+ OT-I cells. (**f**) The percentages of infiltrating GFP^+ OT-I cells in cell suspensions from the ear skin of three mice each at 5 and 14 days after transfer of 5×10^6 GFP^+ OT-I cells. GFP, green fluorescent protein; K14, keratin 14; LN, lymph node; mOVA, membrane-associated ovalbumin; SDLN, skin-draining lymph node; WT, wild type.

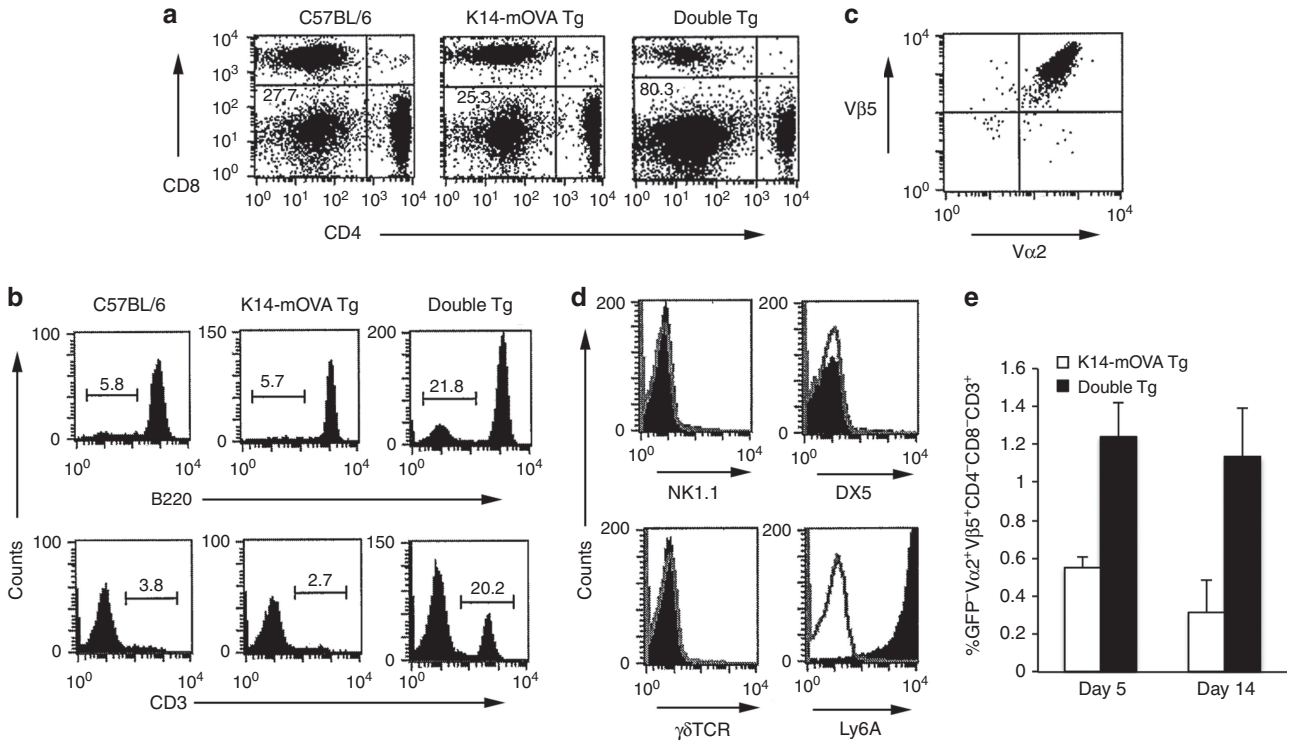

Figure 2. Increase in double negative (DN) T cells in skin-draining lymph nodes (SDLNs) and skin of double transgenic (Tg) mice. (**a**) SDLN cells were harvested from the mice and stained with CD4 and CD8. The numbers in the lower left quadrants indicate the percentages of DN cells in SDLNs. (**b**) The graphs show expression of B220 or CD3 in the gated CD4$^-$CD8$^-$ DN populations. (**c**) DN T cells were purified from pooled lymph nodes (LNs; inguinal LNs, axillary LNs, and mesenteric LNs) and spleens of double Tg mice and stained with CD3, Vα2, and Vβ5. Most of the gated CD3$^+$ cells were Vα2$^+$Vβ5$^+$. (**d**) Vα2$^+$Vβ5$^+$ gated cells stained with various antibodies. The Vα2$^+$Vβ5$^+$ gated cells were stained positively for Ly6A. Thin lines represent isotype controls. (**e**) Percentages of infiltrating GFP$^-$Vα2$^+$Vβ5$^+$CD4$^-$CD8$^-$CD3$^+$ cells in the cell suspensions from ears of three mice each at 5 and 14 days after transfer of 5×10^6 GFP$^+$OT-I cells. GFP, green fluorescent protein; K14, keratin 14; mOVA, membrane-associated ovalbumin.

mice, DN cells were found to be significantly increased in SDLNs of double Tg mice (>80%) compared with DN cells in C57BL/6 and K14-mOVA mice (25%; Figure 2a). The DN cells from C57BL/6 and K14-mOVA Tg mice are mainly B cells expressing B220. In contrast, an additional population of DN cells not expressing B220 was detected in LNs of double Tg mice (Figure 2b). These DN cells included a large population expressing CD3 (DN T cells; 20.2%; Figure 2b). We also identified a population of DN T cells in the spleens of double Tg mice (data not shown).

DN T cells purified from the LNs and spleens of double Tg mice exhibited the Vα2$^+$Vβ5$^+$ TCR as is present in OT-I cells (Figure 2c), but not NK1.1, DX5, or γδ TCR (Figure 2d). Consistent with Zhang *et al.* (2002), who demonstrated that Ly-6A (Sca-1) is highly expressed on DN Tregs, we also found that Ly-6A was highly expressed on DN T cells from double Tg mice (Figure 2d). Thus, adoptively transferred OT-I cells may be regulated by the DN T cells in LNs and spleens. Interestingly, we found that the percentage of DN T cells was significantly increased in the skin of double Tg mice compared with cell suspensions from skin of K14-mOVA mice after adoptive transfer of GFP$^+$OT-I cells (Figure 2e). These findings indicate that migrated OT-I cells may be regulated by DN T cells localized in the skin.

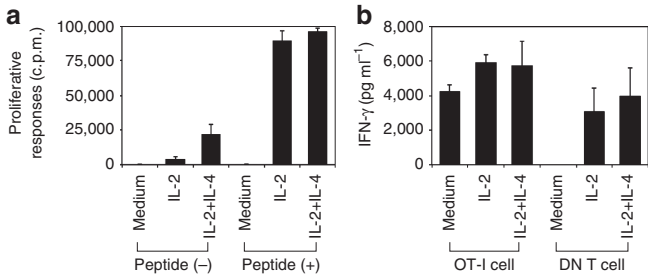

Figure 3. Double negative (DN) T cells from lymph nodes (LNs) and spleen of double transgenic (Tg) mice respond to the ovalbumin (OVA) peptide. (**a**) DN T cells (1×10^5) were stimulated with antigen-presenting cells (APCs; 1×10^5) in the presence or absence of 5 μg ml^{-1} OVA peptide. The DN T cells proliferate when cultured with OVA peptide in the presence of IL-2 alone or IL-2 plus IL-4. (**b**) Production of IFN-γ from OT-I cells and DN T cells was assessed by ELISA. IFN-γ is produced by DN T cells with OVA peptide only in the presence of IL-2 alone or IL-2 plus IL-4. (**a**, **b**) Data are representative of three independent experiments with triplicates in each experiment (error bars, SD).

DN T cells proliferate and produce IFN-γ in the presence of OVA peptide and IL-2

To analyze the capacity of the peripheral DN T cells to proliferate and produce cytokines after Ag stimulation, they were purified from double Tg mice. As shown in Figure 3a,

they did not proliferate in response to OVA peptide without IL-2. However, strong proliferation was observed in response to Ag when IL-2 was added at the start of culture. This response was augmented by the addition of IL-4 to the cultures. Cytokine production by these cells was also assessed (Figure 3b and Supplementary Table S1 online), but no cytokine was detected from culture supernatants of the DN T cells stimulated by Ag in the absence of IL-2. When IL-2 was added, the DN T cells produced IFN-γ (Figure 3b). IL-2, IL-4, and IL-10 production was also assessed using DN T cells, as well as OT-I cells. The latter, when stimulated with IL-2 and IL-4, produced significant amounts of IL-10, suggesting that DN T cells may exert their suppressive effects by producing IL-10 (Supplementary Table S1 online). These results suggest that DN T cells can respond in an Ag-specific manner but require an exogenous source of IL-2 to maintain their viability and their capacity to produce cytokines.

Activated DN T cells do not upregulate cell surface CD8 when cultured with IL-2

To further characterize the DN T cells, the expression of cell surface markers was examined. Purified DN T cells from double Tg mice expressed high levels of CD62L, and they did not express the acute activation markers CD25 and CD69. The cells also expressed low amounts of CD44 (Figure 4a), indicating that they have a naive phenotype. After stimulation with OVA peptide, the number of DN T cells expanded over 20-fold during 10 days of culture (data not shown). The expanded DN T cells expressed CD25, CD69, and CD44, and exhibited reduced expression of CD62L, indicating their activated status after specific Ag stimulation (Figure 4b). The activated DN T cells did not upregulate CD8, and retained the $CD4^-CD8^-$ double-negative phenotype with partially downregulated expressions of CD3, Vα2, and Vβ5 at 10 days after stimulation (Figure 4b), as well as at 2 and 7 days after stimulation (data not shown). We also examined OVA-specific cytotoxicity of the activated DN T cells with a killing assay. DN T cells could not kill EG7 cells, the EL-4 thymoma cells transfected to endogenously produce the SIINFEKL peptide of OVA (Figure 4c). However, $CD8^+$ OT-I cells were able to kill EG7 cells using the same experimental conditions. These results indicate that the DN T cells do not become cytotoxic $CD8^+$ lymphocytes even after stimulation with OVA peptide.

DN T cells are able to suppress proliferation of OT-I cells

It has been demonstrated that DN T cells possess regulatory function and can suppress immune responses mediated by $CD8^+$ or $CD4^+$ T cells that are syngeneic to the DN T cells (Zhang *et al.*, 2000). To determine whether the DN T cells from our double Tg mice have the ability to regulate syngeneic

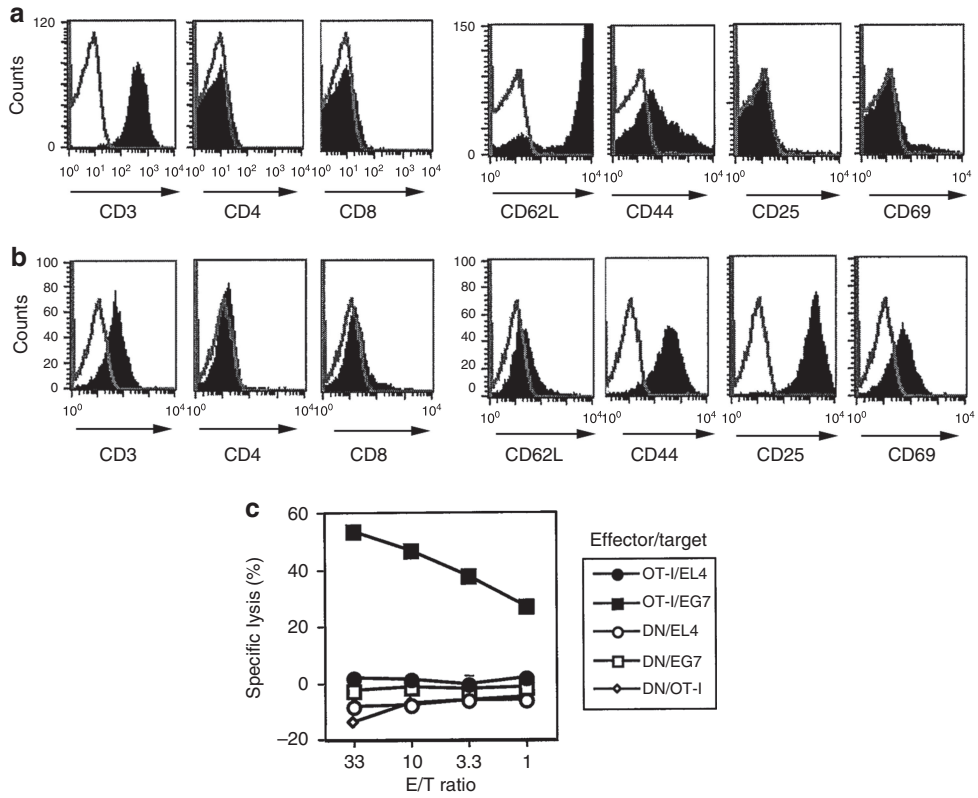

Figure 4. Cultured double negative (DN) T cells with IL-2 are activated and retain the DN phenotype. After 10 days of culture, the DN T cells were harvested and analyzed. Figures illustrate expression of various markers on DN T cells gated on $V\alpha2^+V\beta5^+$ cells in the (**a**) absence or (**b**) presence of peptide. Thin lines represent isotype control staining. (**c**) OT-I or DN T cells, activated by 5-day *in vitro* culture with ovalbumin (OVA) peptide, IL-2, and IL-4 were used as effector cells. OT-I target cells were activated with Concanavalin A and IL-2 for 2 days. Target cells (EL4, EG7, or OT-I cells) were labeled with calcein and incubated with effector cells. OT-I cells exhibited cytotoxicity in a dose-dependent manner. E/T, effector/target cell ratio.

CD8$^+$ T cells (OT-I cells), carboxyfluorescein succinimidyl ester (CFSE)–labeled OT-I cells were stimulated with OVA peptides and cultured with or without DN T cells that were isolated from double Tg mice and activated *in vitro*. CFSE-labeled OT-I cells were reduced in the wells containing DN T cells (Figure 5). This reduction of OT-I cells indicated that the DN T cells may kill OT-I cells. When the number of DN T cells in the culture wells was increased, suppression of proliferation of OT-I cells was also observed. These results suggest that the DN T cells are effective regulators of OT-I cells. The inability of DN T cells, activated *in vitro*, to suppress GVHD (Supplementary Figure S2 online) may be due to their undergoing apoptosis shortly after adoptive transfer.

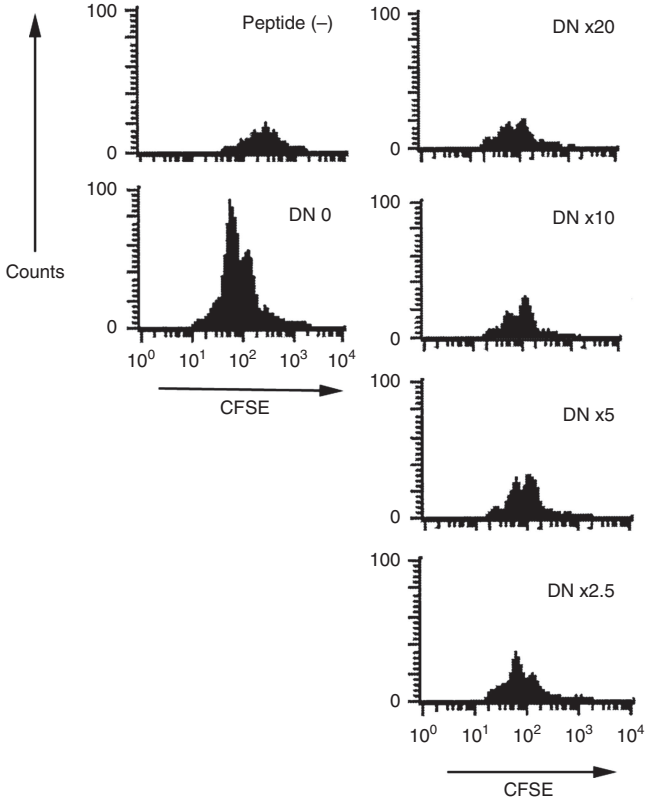

Figure 5. Regulatory function(s) of double negative (DN) T cells from double transgenic (Tg) mice. Naive OT-I cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with activated DN T cells, and proliferative responses in the presence of antigen were assessed by flow cytometry. Figures show gated CD8$^+$CFSE$^+$ cells. Ratios of DN T cells to OT-I cells are indicated as DN ×20 to ×2.5.

DN T cells specifically kill syngeneic CD8$^+$ T cells

To determine whether DN T cells isolated from double Tg mice could kill OT-I cells, DN T cells and OT-I cells were used as effector cells and target cells, respectively, in a calcein-release killing assay to detect a perforin-dependent cytolytic pathway. However, DN T cells did not kill OT-I cells (Figure 4c). Next, we performed a JAM test using activated OT-I cells labeled with [^{3}H]-thymidine as target cells to detect a Fas-dependent pathway. Retention of [^{3}H]-thymidine by target OT-I cells was inhibited by effector DN T cells in an effector/target cell ratio–dependent manner (Figure 6a). The killing of OT-I cells by DN T cells was blocked by the addition of Fas/Fc fusion protein before and during the JAM test (Figure 6b). These results indicate that a Fas/Fas ligand (FasL) interaction is involved in DN T cell–mediated cytotoxicity of OT-I cells. We next determined the Ag specificity of DN T cell–mediated cytotoxicity. On the other hand, when activated Matahari cells that express a TCR with a different Ag specificity from DN T cells were used as targets, DN cells were not cytotoxic (Figure 6c). Consistent with a previous report (Zhang *et al.*, 2000), our data demonstrate that DN T cells are able to kill activated CD8$^+$ T cells through a Fas-dependent pathway, and that sharing of the TCR specificity by both DN T cells and target cells is required for cytotoxicity to occur.

DISCUSSION

To understand the mechanisms of autoimmunity and tolerance against skin-associated self-Ag, we developed transgenic mice that express membrane-tethered or soluble form of a model self-Ag, OVA, under the control of a K14 promoter (Miyagawa

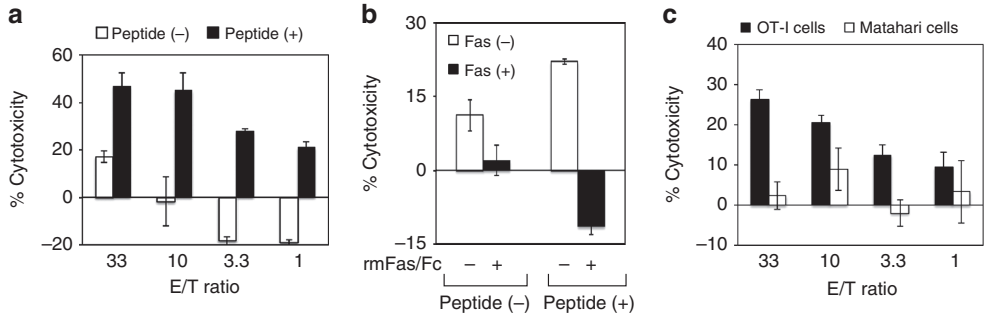

Figure 6. Double negative (DN) T cells specifically kill activated CD8$^+$ T cells through the Fas/Fas ligand (FasL) pathway. (**a**) OT-I cells were activated by anti-CD3 mAb, labeled with [^{3}H]-thymidine, and used as target cells (1.5×10^4 per well). They were pulsed with the ovalbumin (OVA) peptide or left untreated. DN T cells were incubated with labeled target cells for 18 hours and exhibited cytotoxicity in a dose-dependent manner. (**b**) Cytotoxicity is inhibited in the presence of a Fas/Fc fusion protein at an effector/target cell (E/T) ratio of 20:1. (**c**) OT-I cells and Matahari cells were stimulated independently with anti-CD3 mAb. DN T cells were stimulated with the OVA peptide. Activated DN T cells were cytotoxic to OT-I cells but not to H-Y peptide-pulsed Matahari cells. (**a**–**c**) Data are representative of three independent experiments with triplicates in each experiment (error bars, SD).

et al., 2010). Using these mice, we have identified several factors that induce tolerance or attenuate autoimmune responses (Miyagawa *et al.*, 2008; Gutermuth *et al.*, 2009; Miyagawa *et al.*, 2012; Paek *et al.*, 2012). In this study, we report that TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$DN Tregs that are present in the LNs, spleens, and skin may be potential regulatory cells to control the tolerance status in double Tg mice. We have demonstrated that double Tg mice expressing the transgenes for both OT-I TCR and K14-mOVA develop tolerance to the OVA Ag, and that DN T cells, which are increased in double Tg mice, have Ag-specific regulatory functions. DN T cells from double Tg mice suppress the proliferation of CD8$^+$ OT-I cells that have the same TCR specificity as DN T cells. We also demonstrated that DN T cells selectively kill activated OT-I cells via a Fas/FasL–dependent mechanism. In addition, DN T cells may exert their suppressive effects by producing IL-10. Ideally, the ability of DN T cells could be functionally assessed by *in vivo* depletion from tolerant double Tg mice or by adoptive transfer into K14-mOVA Tg mice. However, owing to the lack of specific markers on DN T cells, selective depletion of DN T cells is currently not possible *in vivo* without affecting other T-cell subsets. Similarly, the purification of a sufficient number of DN T cells from double Tg mice for adoptive transfer is not possible, because only small numbers of these cells can be purified. Instead, we transferred DN T cells expanded *in vitro* together with OT-I cells into K14-mOVA Tg mice to determine whether DN T cells suppress GVHD. The results showed that the activated DN T cells did not suppress GVHD (Supplementary Figure S2 online), a finding that is not surprising, as injection of activated cells may not mimic injection of nonactivated DN T cells. Moreover, it is likely that most activated DN T cells die after adoptive transfer into K14-mOVATg mice.

Ag-specific DN Tregs were first identified and characterized by Zhang *et al.* (2000). They demonstrated that DN Tregs have an important role in the induction of tolerance in transplantation murine models of allogeneic skin (Zhang *et al.*, 2000; Ford *et al.*, 2002) and of xenogeneic heart (Chen *et al.*, 2003). Since then, several studies have demonstrated that DN Tregs exhibit immune suppressive effects in an Ag-specific manner in multiple disease models both *in vitro* and *in vivo* (Chen *et al.*, 2005; Ford *et al.*, 2007; Zhang *et al.*, 2007; Hillhouse *et al.*, 2010; Young *et al.*, 2003; McIver *et al.*, 2008; Dugas *et al.*, 2010). DN Treg cells have been reported to be present in humans (Fischer *et al.*, 2005), and comprise 1–3% of peripheral T cells in normal mice and humans (Zhang *et al.*, 2000; Fischer *et al.*, 2005).

DN Tregs can exhibit cytotoxicity through various mechanisms depending on the type of target cells. They have been demonstrated to kill syngeneic CD8$^+$ and CD4$^+$ T cells in an Ag-specific manner through Fas/FasL interaction (Zhang *et al.*, 2000; Ford *et al.*, 2002). Other studies have shown that DN Tregs kill autologous B cells via the perforin/granzyme pathway (Zhang *et al.*, 2007), whereas they kill alloantigen-expressing dendritic cells mainly through Fas/FasL interaction (Gao *et al.*, 2011). Our study also demonstrated that DN T cells kill syngeneic CD8$^+$OT-I cells through the Fas/FasL-dependent pathway but not through the perforin/granzyme pathway.

Although the role of DN Tregs in preventing allo- or xeno-immune responses is well studied, their role in preventing T cell–mediated autoimmune disease is not well known. Few studies have addressed this by using self-Ag Tg murine models. Priatel *et al.* (2001) suggested that DN T cells bearing the alloreactive H-2^b 2C TCR in Ag-expressing mice had regulatory function and could suppress autoreactive CD8$^+$ T-cell proliferation by using a Fas/FasL-dependent cytolytic mechanism. Subsequently, Ford *et al.* (2007) demonstrated that injection of gp33 peptide prevented the development of type 1 diabetes in gp33 peptide–specific TCR Tg (P14) mice with increased DN T cells and decreased CD8$^+$ T cells. These gp33 peptide–activated DN T cells suppressed proliferation of syngeneic CD8$^+$ T cells via their cytotoxicity in an Ag-specific manner. Our K14-mOVA Tg murine model shows that the proportion of DN T cells is increased in peripheral lymphoid organs, as well as in the skin in tolerant double Tg mice. The presence of OT-I cells and DN T cells in the skin of double Tg mice indicates that some OT-I cells escape from regulation in the peripheral lymphoid organs and migrate to the skin, but may be further regulated by local DN T cells. It is unclear as to which regulatory mechanism dominates in these tolerant double Tg mice. The data suggest that DN T cells may provide a novel therapeutic approach to Ag-specific T cell–mediated autoimmune diseases.

MATERIALS AND METHODS

Mice

All mice were obtained from the National Cancer Institute Animal Production Program (Frederick, MD), housed in a clean facility, and bred and used in accordance with the institutional guidelines. OT-I mice were obtained from Dr Judy Kapp (Emory University, Atlanta, GA). Rag-1-deficient OT-I mice were purchased from Taconic (Germantown, NY). GFP$^+$OT-I Tg mice were generated by crossing human ubiquitin C promoter-GFP Tg mice (Jackson, ME) with OT-I mice. K14-mOVA Tg mice have been described previously (Shibaki *et al.*, 2004). Double Tg mice were generated by crossing K14-mOVA Tg mice with OT-I mice.

Adoptive transfer

Five million OT-I cells or GFP$^+$OT-I cells in 200 μl of phosphate-buffered saline were injected intravenously into K14-mOVA mice or double Tg mice. Weight and health status were monitored daily for 14 consecutive days after injection.

Antibodies and flow cytometry

FITC-conjugated anti-CD3, B220, NK1.1, γδTCR, Vα2, phycoerythrin-conjugated anti-CD4, CD8, CD3, CD62L, CD25, CD69, CD44, DX5, Ly6A, Vβ5, PerCP5.5-conjugated anti-CD8, allophycocyanin-conjugated anti-CD8, Alexa700-conjugated anti-Vα2, allophycocyanin-Cy7-conjugated anti-CD4, and biotin-conjugated anti-Vβ5 (BD Bioscience, San Jose, CA) mAbs were used for cell surface analysis. Allophycocyanin-conjugated streptavidin was used for the visualization of biotin-conjugated mAb. Stained cells were analyzed on FACSCalibur and LSR II flow cytometers (BD Bioscience).

Preparation of OT-I cells and Matahari cells

Single-cell suspensions were prepared from pooled LNs and spleens of OT-I mice, the GFP/OT-I mice. $CD8^+$ cells were purified by Mouse CD8 T-cell columns (R&D System, Minneapolis, MN). In some experiments, single-cell suspensions from LNs of Rag-1-deficient OT-I mice were used as $CD8^+$OT-I cells.

Purification of $CD3^+CD4^-CD8^-$ DN T cells

DN T cells were purified from pooled LNs (inguinal LNs, axillary LNs, and mesenteric LNs) and spleens of 6- to 12-week-old double Tg mice as follows. The lymphocytes were passed through Mouse T-cell enrichment columns (R&D Systems) and stained with FITC-conjugated anti-CD4, CD8, and B220 mAbs. The cells were then incubated with anti-FITC MicroBeads (Miltenyi Biotec, Auburn, CA) and purified by negative selection. The purity of DN T cells was routinely 90–98%.

Stimulation of T cells

Purified DN T cells were cultured at a density of 1×10^6 cells per well in a 24-well plate in the presence of 2×10^6 irradiated (3,000 rad) and sex-matched splenocytes from C57BL/6 mice pulsed with $5\,\mu g\,ml^{-1}$ OVA peptide (SIINFEKL) as antigen-presenting cells, $50\,ng\,ml^{-1}$ recombinant mouse IL-2, and $30\,ng\,ml^{-1}$ recombinant mouse IL-4 (Peprotech, Rocky Hill, NJ) in 10% fetal bovine serum, 10 mM MEM nonessential amino acids, 1 mM sodium piruvate, 10 mM HEPES, and penicillin/streptomycin RPMI-1640 culture medium (complete RPMI) for various times. Activated cells were then used for experiments. One million GFP^+OT-I cells/well in 24-well plates were cocultured with 1×10^6 splenocytes from C57BL/6 mice treated with $1\,ng\,ml^{-1}$ SIINFEKL and $50\,mg\,ml^{-1}$ mitomycin C (Sigma-Aldrich, St Louis, MO) in the presence of $20\,ng\,ml^{-1}$ recombinant mouse IL-2 in complete RPMI for 5 days. The activated GFP^+OT-I cells were used for adoptive transfer experiments.

Collection of skin-infiltrating cells

Ears were minced and digested in RPMI-1640 containing $2\,mg\,ml^{-1}$ Liberase TL (Roche Diagnostics, Indianapolis, IN) at 4 °C overnight and were then incubated at 37 °C for 30 minutes in RPMI-1640 containing $2\,mg\,ml^{-1}$ Liberase TL and $0.04\,mg\,ml^{-1}$ DNase I (Sigma-Aldrich) under gentle agitation. The suspensions were dissociated by pipetting and were sequentially passed through 70- and 100-μm filters.

Proliferation assays

DN T cells (1×10^5) were stimulated with antigen-presenting cells (1×10^5) in the presence or absence of $5\,\mu g\,ml^{-1}$ OVA peptide in a 96-well flat-bottom plate. [^{3}H]-thymidine was added during the last 16 hours of a 3.5-day culture.

ELISA

DN T cells (1×10^5) or OT-I cells (1×10^5) were stimulated with $5\,\mu g\,ml^{-1}$ OVA peptide and antigen-presenting cells (1×10^5). Supernatants were removed 48 hours later and assayed using ELISA kits (R&D Systems).

CFSE labeling

OT-I cells (1×10^7/ml) were labeled with 2 μM CFSE (Molecular Probes, Eugene, OR) in phosphate-buffered saline for 10 minutes at 37 °C. Cells were then washed and resuspended in complete RPMI.

Suppression assay

DN T cells were cultured for 5 days and harvested. Varying numbers of DN T cells (DN T cells: CFSE-labeled OT-I cells was 2.5–20:1.) were mixed with CFSE-labeled OT-I cells. After a 2-day incubation, all the cells in each well were harvested and analyzed by flow cytometry. Cultures without DN T cells were used as controls.

Killing assays

A calcein AM release assay was used to determine perforin-dependent cytotoxicity. OT-I cells or DN T cells stimulated for 3–5 days were used as effector cells. As target cells, OT-I cells were activated with Concanavalin A and IL-2 for 2 days. A thymoma cell line, EL4, and OVA-expressing EL4 (EG7) were treated with $500\,U\,ml^{-1}$ IFN-γ (Peprotech) for 2 days and used as target cells. Target cells were then labeled with calcein AM (Molecular Probes) and added to 96-well round-bottom plates (1.5×10^4/well) with specific ratios of effector cells for 3 hours at 37 °C. The supernatants were recovered, and calcein AM release was measured using a CytoFluor 2,350 plate reader (Millipore, Billerica, MA). Specific lysis = ((experimental − spontaneous)/(maximal − spontaneous)) × 100. Maximal lysis was achieved with 0.1% Triton X-100, and spontaneous lysis was obtained by incubating target cells alone. The JAM test was used to determine Fas-dependent cytotoxicity of DN T cells. DN T cells stimulated for 3 days were harvested and seeded into 96-well round-bottom plates as effector cells. The target cells (OT-I cells and Matahari cells) were stimulated with plate-bound purified anti-CD3 mAb ($10\,\mu g\,ml^{-1}$; BD Bioscience), and $10\,\mu Ci\,ml^{-1}$ of [^{3}H]-thymidine was added during the last 18 hours of a 2-day culture. [^{3}H]-thymidine–labeled target cells were pulsed with peptides for 1 hour, and then cocultured with the effector cells at 37 °C for 18 hours. The cells were harvested and counted in a β-counter. Specific cell lysis was calculated using the following equation: percentage of specific killing = $(S - E)/S \times 100$, in which E (experimental) is the c.p.m. of retained DNA in the presence of effector cells, and S (spontaneous) is the c.p.m. of retained DNA in the absence of effector cells. In some experiments, DN T cells were treated with $10\,\mu g\,ml^{-1}$ Fas/Fc fusion protein (R&D Systems) to block FasL on DN T cells. Fas/Fc fusion protein was added 1 hour before and during the killing assays.

Histological analysis

Tissue samples were fixed in 10% neutral-buffered formalin. Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin staining using standard techniques (American HistoLabs, Rockville, MD).

Statistical analysis

Data were compared using Student's t-test. Values of $P < 0.05$ were referred to as a significant difference.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Jay Linton for excellent technical assistance.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Bouneaud C, Kourilsky P, Bousso P (2000) Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity* 13:829–40

Chen W, Ford MS, Young KJ *et al.* (2003) Role of double-negative regulatory T cells in long-term cardiac xenograft survival. *J Immunol* 170:1846–53

Chen W, Zhou D, Torrealba JR *et al.* (2005) Donor lymphocyte infusion induces long-term donor-specific cardiac xenograft survival through activation of recipient double-negative regulatory T cells. *J Immunol* 175:3409–16

Duarte N, Stenstrom M, Campino S *et al.* (2004) Prevention of diabetes in nonobese diabetic mice mediated by CD1d-restricted nonclassical NKT cells. *J Immunol* 173:3112–8

Dugas V, Beauchamp C, Chabot-Roy H *et al.* (2010) Implication of the CD47 pathway in autoimmune diabetes. *J Autoimmun* 35:23–32

Fischer K, Voelkl S, Heymann J *et al.* (2005) Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells. *Blood* 105:2828–35

Ford MS, Chen W, Wong S *et al.* (2007) Peptide-activated double-negative T cells can prevent autoimmune type-1 diabetes development. *Eur J Immunol* 37:2234–41

Ford MS, Young KJ, Zhang Z *et al.* (2002) The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. *J Exp Med* 196:261–7

Gao JF, McIntyre MS, Juvet SC *et al.* (2011) Regulation of antigen-expressing dendritic cells by double negative regulatory T cells. *Eur J Immunol* 41:2699–708

Gonzalez A, Andre-Schmutz I, Carnaud C *et al.* (2001) Damage control, rather than unresponsiveness, effected by protective DX5+ T cells in autoimmune diabetes. *Nat Immunol* 2:1117–25

Gutermuth J, Nograles KE, Miyagawa F *et al.* (2009) Self-peptides prolong survival in murine autoimmunity via reduced IL-2/IL-7-mediated STAT5 signaling, CD8 coreceptor, and V alpha 2 down-regulation. *J Immunol* 183:3130–8

Hayday A, Tigelaar R (2003) Immunoregulation in the tissues by gamma/delta T cells. *Nat Rev Immunol* 3:233–42

Hillhouse EE, Beauchamp C, Chabot-Roy G *et al.* (2010) Interleukin-10 limits the expansion of immunoregulatory CD4-CD8- T cells in autoimmune-prone non-obese diabetic mice. *Immunol Cell Biol* 88:771–80

Jiang H, Chess L (2000) The specific regulation of immune responses by CD8+ T cells restricted by the MHC class Ib molecule, Qa-1. *Annu Rev Immunol* 18:185–216

Jones LA, Chin LT, Longo DL *et al.* (1990) Peripheral clonal elimination of functional T cells. *Science* 250:1726–9

Kappler JW, Roehm N, Marrack P (1987) T cell tolerance by clonal elimination in the thymus. *Cell* 49:273–80

Kisielow P, Bluthmann H, Staerz UD *et al.* (1988) Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature* 333:742–6

McIver Z, Serio B, Dunbar A *et al.* (2008) Double-negative regulatory T cells induce allotolerance when expanded after allogeneic haematopoietic stem cell transplantation. *Br J Haematol* 141:170–8

Miyagawa F, Tagaya Y, Kim BS *et al.* (2008) IL-15 serves as a costimulator in determining the activity of autoreactive CD8 T cells in an experimental mouse model of graft-versus-host-like disease. *J Immunol* 181:1109–19

Miyagawa F, Gutermuth J, Zhang H *et al.* (2010) The use of mouse models to better understand mechanisms of autoimmunity and tolerance. *J Autoimmun* 35:192–8

Miyagawa F, Zhang H, Terunuma A *et al.* (2012) Interferon regulatory factor 8 integrates T-cell receptor and cytokine-signaling pathways and drives effector differentiation of CD8 T cells. *Proc Natl Acad Sci USA* 109:12123–8

Paek SY, Miyagawa F, Zhang H *et al.* (2012) Soluble Peptide Treatment Reverses CD8 T-Cell-Induced Disease in a Mouse Model of Spontaneous Tissue-Selective Autoimmunity. *J Invest Dermatol* 132:677–86

Pircher H, Burki K, Lang R *et al.* (1989) Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* 342:559–61

Priatel JJ, Utting O, Teh HS (2001) TCR/self-antigen interactions drive double-negative T cell peripheral expansion and differentiation into suppressor cells. *J Immunol* 167:6188–94

Rocha B, von Boehmer H (1991) Peripheral selection of the T cell repertoire. *Science* 251:1225–8

Sakaguchi S, Yamaguchi T, Nomura T *et al.* (2008) Regulatory T cells and immune tolerance. *Cell* 133:775–87

Sharif S, Arreaza GA, Zucker P *et al.* (2002) Regulation of autoimmune disease by natural killer T cells. *J Mol Med (Berl)* 80:290–300

Shevach EM (2011) Biological functions of regulatory T cells. *Adv Immunol* 112:137–76

Shibaki A, Sato A, Vogel JC *et al.* (2004) Induction of GVHD-like skin disease by passively transferred CD8(+) T-cell receptor transgenic T cells into keratin 14-ovalbumin transgenic mice. *J Invest Dermatol* 123:109–15

Voelkl S, Gary R, Mackensen A (2011) Characterization of the immunoregulatory function of human TCR-alphabeta+ CD4- CD8- double-negative T cells. *Eur J Immunol* 41:739–48

Walker LS, Abbas AK (2002) The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol* 2:11–9

Young KJ, DuTemple B, Phillips MJ *et al.* (2003) Inhibition of graft-versus-host disease by double-negative regulatory T cells. *J Immunol* 171:134–41

Young KJ, Zhang L (2002) The nature and mechanisms of DN regulatory T-cell mediated suppression. *Hum Immunol* 63:926–34

Zhang D, Yang W, Degauque N *et al.* (2007) New differentiation pathway for double-negative regulatory T cells that regulates the magnitude of immune responses. *Blood* 109:4071–9

Zhang ZX, Stanford WL, Zhang L (2002) Ly-6A is critical for the function of double negative regulatory T cells. *Eur J Immunol* 32:1584–92

Zhang ZX, Yang L, Young KJ *et al.* (2000) Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med* 6:782–9