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Selective Elimination of Autoreactive T cells in vivo by the Regulatory T Cells

Xing Chang, Pan Zheng, and Yang Liu

Division of Immunotherapy, Department of Surgery, Comprehensive Cancer Center and Program of Molecular Mechanism of Diseases, University of Michigan, Ann Arbor, MI 48109

Summary

How regulatory T cells (Treg) control autoreactive T cells has not been analyzed in animals with a normal T cell repertoire. Using endogenous viral superantigens (VSAg) as the primary self antigens and mice with the *Scurfy* mutation of *FoxP3*, we show here that the Treg defect causes preferential accumulation of autoreactive T cells. Interestingly, in the *Scurfy* mice, the proliferation of VSAg-reactive T cells was no more vigorous than that of non-VSAg-reactive T cells, which indicated that the preferential accumulation is not due to preferential proliferation. In contrast, VSAg-reactive T cells disappears in WT host despite their preferential proliferation. Importantly, when adoptively transferred into the newborn *Scurfy* mice, the Treg selectively kill autoreactive T cells without affecting their proliferation. The selective elimination is due to increased susceptibility of autoreactive T cells to Treg-mediated killing.

Keywords

autoimmune diseases; clonal deletion; FoxP3; viral super-antigen; regulatory T cells

Introduction

It is well established that Treg play a critical role in controlling autoimmune diseases [1]. However, the mechanisms by which Treg control autoreactive T cells are largely unclear [2]. While Treg activity is classically measured by their repression of T cell proliferation in vitro [3], their roles in controlling T cell proliferation has been questioned by several lines of evidence. First, Chen et al [4] reported that, when autoreactive TCR transgenic mice were rendered defective in FoxP3 gene and hence devoid of Treg, the activation in the lymphoid organ and recruitment of the autoreactive T cells were unaffected. However, the infiltrating T cells were immediately destructive in the target organ. These data suggest that the primary function of Treg is to regulate the effector function of T cells in the target tissue. Second, using multiphoton intravital microscopy, Mempel et al. [5] showed that Treg induced a reversible defect in the cytotoxic T lymphocytes (CTL) in target cell killing, although Treg caused no defect in proliferation, induction of cytotoxic effector molecules and secretory granules, in situ motility, or ability to form antigen-dependent conjugates with target cells.

Correspondence to: Yang Liu, yangl@umich.edu.

Authorship XC designed and performed experiments and wrote the manuscript. PZ and YL participated in experimental design, data analysis and writing the manuscript.

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Since both studies were done using transgenic T cells, it would be of great interest to determine how Treg regulate autoreactive T cells in mice with a normal T cell repertoire. This effort has been hampered by difficulties in studying the behavior of self-reactive Treg and effector cells in models other than TCR transgenic mice. An exception to this rule is VSAg, which are encoded by murine mammary tumor viruses (MMTV) [6]. In strains that have genomic insertion by endogenous MMTV, the VSAg has served as model antigens to provide the first demonstration of clonal deletion, as the specificity is encoded by the V β region [7]. In this study, we analyzed the effect of Treg on VSAg-reactive T cells using mice with a mutation of *FoxP3*, a master regulator for the development and function of Treg [8;9;10]. Our data demonstrate that Treg selectively eliminate autoreactive T cells because the latter are more susceptible to killing by Treg.

Materials and Methods

Animals

Thy1.1 BALB/c mice with mutation of *FoxP3* (*FoxP3^{sf}*) were produced after more than 12 generations of backcross in the University of North Carolina. The genotype of scurfy mice were determined through allele specific PCR as described [11]. Wild type BALB/c mice were obtained through a subcontract of National Cancer Institute. B10.BR mice were purchased from Jackson Laboratories. All the mice were maintained under specific pathogen-free conditions at the University Laboratory Animal Resources at the Ohio State University for the duration of the study.

BrdU incorporation and measurements

Mice were injected i.p. with nucleotide analog bromodeoxyuridine (BrdU; 1 mg/mouse in 100 μ l PBS) 3 h before sacrifice. Splenocytes and lymph node cells were prepared and BrdU incorporation was detected by flow cytometry with a BrdU Flow Kit in conjunction with other cell surface markers, as described by the manufacturer (BD Biosciences, La Jolla, CA).

Antibodies and flow cytometry

Single cell suspension of thymus, spleen or lymph nodes were prepared and first blocked with anti-FcR (2.4G2) to eliminate Fc-mediated non-specific bindings. For cell surface staining, samples were stained with antibodies on ice for 30 minutes in staining buffer and were fixed by 1% PFA. Intracellular staining of the FoxP3 was performed as described by the manufacturer (eBiosciences, La Jolla, CA). The following antibodies were used: FITC or PE conjugated antibodies against TCR V β 3, V β 5, V β 8, V β 11, V β 12 (BD biosciences), Percp cy5.5 conjugated anti-CD4 and anti-CD8 (BD Biosciences), APC-conjugated anti-CD4, anti-CD8 and anti-Thy1.2 (eBiosciences), PE-conjugated anti-CD25 (PC61) and anti-Foxp3 (FJK-16) (eBiosciences). All samples were analyzed by a four color FACS Caliber (BD biosciences).

For the Annexin V staining, cells were first stained with cell surface antibodies and then were incubated with PE conjugated Annexin V (BD Biosciences) at room temperature for 15 min and were analyzed by FACS immediately after the staining.

Cell purification and adoptive transfer

To purify CD4⁺CD25⁺ cells, CD4⁺ T cells were first purified using the Dynal beads to remove non-CD4 cells and then CD25⁺CD4⁺ T cells were further purified using the MACS beads. Briefly, spleen and lymph node cells from 6-8 weeks old BALB/C mice were first incubated with anti-FcR (2.4 G2), anti-CD8 (2.4.3), anti-CD11b (MAC-1), anti-B220, and N418 (anti-CD11c) antibodies. The antibody-coated cells were then depleted with anti-Rat IgG-coated magnetic beads (Dynal, Invitrogen) were used to deplete. Purified CD4 T-cells were stained

with anti-CD25 PE followed by anti-PE MACS beads (Miltenyi Biotec, Auburn, CA), CD4⁺CD25⁺ cells were then positively selected using MACS LS columns. Then purity of CD4⁺CD25⁺ cells was routinely around 92% to 95%. 1 million purified CD4⁺CD25⁺ cells were resuspended in serum free RPMI and i.v injected into 2-3 days old Thy1.1 BALB/c scurfy mice and their wild type littermates.

In vitro cytotoxicity of regulatory T cells

CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were stimulated with 10 µg/ml of plate-bound anti-CD3 and 100U/ml of IL-2 for 72 hours. These pre-activated T cells were then mixed with fresh lymph nodes cell from 8-10 days old scurfy mice in 1:1 ratio for 4 hours. The cells were then surface-stained with APC conjugated anti-Thy1.1, PE-conjugated anti-CD4 and FITC-conjugated anti-TCR Vβ5 or Vβ8. After the surface staining, 7-AAD was added to each sample which was then analyzed by FACS immediately. Thy1.1⁺CD4⁺ Vβ5⁺ or Thy1.1⁺CD4⁺ Vβ8⁺ were gated as target cells respectively. Death of the target cells was determined by the % of 7-AAD⁺ cells. Specific lysis was calculated by % of 7-AAD⁺ cells with effector cells minus % of 7-AAD⁺ cells in cultures without any effector cells.

Statistic analysis

All the data are shown in Mean+SEM. Two tail student T test were employed and statistic significance is **, P<0.01; *, P< 0.05.

Results

1. Normal clonal deletion of VSAg-reactive T cells in the Scurfy mice

The genome of the BALB/c mice has insertions of mouse mammary tumor provirus (MMTV) type 6, 8 and 9 as well as H-2I-E, which in conjunction formed viral superantigens recognized by T cells expressing Vβ3, 5, 11 and 12 [6]. The VSAg-reactivity of these T cells allows us to follow the fate of the autoreactive T cells by flow cytometry in a polyclonal TCR level manner. We first determined whether mutation of FoxP3 affects clonal deletion of the thymocytes. We stained the thymocytes from the Scurfy mice and their WT littermates with anti-CD4 and CD8 mAbs in conjunction with the mAbs specific for Vβ3, 5, 8, 11 and 12. Representative profiles of CD4 and CD8 single positive thymocytes are shown in Fig. 1a and the summary data are presented in Fig. 1b. Among both CD4 and CD8 T cells, Vβ3, 5, and 12-expressing cells were less than 2% in both WT and the Scurfy mice. In our experience, Vβ11⁺ T cells were not completely deleted in the thymus at this time point and the frequency of Vβ11⁺ T cells was also comparable between WT and the FoxP3 mutant mice. These results demonstrate that the FoxP3 mutation does not affect clonal deletion in the thymus.

Interestingly, in the WT mice, clonal deletion of VSAg reactive T cells resulted in the enrichment of regulatory T cells in those undeleted autoreactive T cells. Thus, the proportion of regulatory T cells was not uniformly distributed among different Vβ-expressing T cells. As shown in Fig. 2, in the thymus about 8% of the Vβ3, 5, 11 and 12-expressing T cells express Foxp3, a lineage marker of regulatory T cells, while only about 3% of the Vβ8⁺ T cells expressed Foxp3. Similarly, in the spleen, more than 30% of VSAg-reactive cells were Foxp3⁺, whereas less than 12% of non-VSAg reactive T cells had FoxP3 expression. Since the relative enrichment of the FoxP3⁺ cells in the VSAg-reactive subsets were similarly in the spleen (2.5) and in the thymus (2.6), the enrichment was not due to peripheral expansion of VSAg-reactive Treg in the spleen. Since the total number and the percentage of VSAg-reactive Treg was not higher than the B6 mice that lack the I-E for presentation of superantigens (Figure 2c) [12], the increase was likely due to the elimination of CD25⁻ T cells rather than positive selection of VSAg-reactive Treg.

2. Increased accumulation of VSAg-reactive T cells in the scurfy mice is not associated with hyper-proliferation of VSAg-reactive T cells

Interestingly, in the scurfy mutant mice a substantial increase in the % of VSAg-reactive T cells was observed in the spleen and lymph nodes in comparison to what was observed in the thymus. As shown in Fig. 3a, b, at 15 days of age, while WT littermates had less than 1% of V β 3 and V β 5-expressing CD4 T cells in the spleen, the Scurfy mice had almost 10-fold more V β 3⁺ or V β 5⁺ CD4 T cells. Likewise, we also observed a significantly higher number of V β 11⁺ or V β 12⁺ CD4 T cells in the Scurfy spleen, although the fold of expansion was not as striking as what was observed for the V β 3⁺ and V β 5⁺ CD4 T cells, perhaps due to higher baseline levels in the WT mice. A similar expansion was also observed for the CD8 T cells (Fig. 3b) and can be found in the lymph nodes (data not shown).

Preferential expansion of the VSAg-reactive T cells in the scurfy mice can be due to either their enhanced proliferation or survival of the autoreactive T cells. To test if the increased proliferation was responsible for their accumulation, we pulsed the scurfy mice and the WT littermates with BrdU for 3 hours and analyzed the % of cells that had undergone proliferation. As shown in Fig. 3c & d, in WT mice, the V β 3, 5, and 12⁺ CD4 T cells divided substantially faster than the V β 8⁺ T cells, which are not VSAg-reactive in the BALB/c mice. The proliferation did not lead to expansion of the VSAg-reactive T cells as their frequencies were no higher than what was found in the thymus. In contrast, in the Scurfy mice, equally rapid BrdU incorporation was observed in the VSAg-reactive and nonreactive subsets. Moreover, the proliferation of V β 3, 5, and 12⁺ T cells was comparable between in Scurfy mice as in their WT littermates. Nevertheless, preferential accumulation of the VSAg-reactive T cells was only found in the Scurfy mice. Since V β 11⁺ did proliferate more in the Scurfy mice, preferential proliferation may account for its preferential accumulation. In 8-day old Scurfy mice (Fig. 3e), when the autoreactive T cell responses were at early phase, the non-VSAg-reactive T cells proliferated at least as fast as that of the VSAg-reactive T cells. Taken together, our data demonstrate that increased proliferation is neither necessary nor sufficient for preferential accumulation of VSAg-reactive T cells in the absence of Treg. As such, the primary function of Treg unlikely to suppress proliferation of autoreactive T cells.

3. Elimination of autoreactive T cells by Treg after their proliferation in the periphery

Since Scurfy mice lack Treg, we tested whether the expansion of VSAg-reactive T cells can be suppressed by Treg. We adoptively transferred Treg isolated from WT mice into 2 or 3 day old Scurfy mice. On day 15, the recipients were pulsed for 3 hours with BrdU prior to sacrifice and analyzed for the frequency and proliferation of both VSAg-reactive and non-reactive T cells. Since the Scurfy mice are of Thy1.1 genotype and the donor Treg were from Thy1.2 BALB/c mice, we were able to follow the fate of both Treg and endogenous VSAg-reactive T cells. As shown in Fig. 4a, the Treg expanded substantially in the Scurfy host in comparison to the WT host, although higher number of Treg was observed in the lymph nodes than the spleen. Moreover, Treg recovered from the Scurfy recipients exhibited more profound down-regulation of CD62L, which indicated that the Treg were activated in the Scurfy recipients. Treg treatment caused reduction in V β 3⁺ and V β 5⁺ T cells in the lymph node and spleen (Fig. 4b, c). The reduction of V β 3⁺ and V β 5⁺ T cells was not due to Treg-mediated suppression of proliferation of VSAg-reactive T cells, as this proliferation was unaffected by adoptive transfer of Treg (Fig. 4d). However, a significant reduction in proliferation of V β 8 and V β 11⁺ cells was observed. These data, together with enhanced proliferation of these subsets in the Scurfy mice (Fig. 3c) demonstrate that Treg inhibits proliferation of some T cells in vivo.

Interestingly, significant reduction of V β 11⁺ and V β 12⁺ T cells was not observed. Lack of effect on V β 11⁺ T cells, together with apparent lack of clonal deletion at this early timepoint

may reflect insufficient self-ligand for this subset, although the lack of effect on $V\beta 12^+$ T cells suggests that other yet unknown factors may be responsible for the discrepancy.

We then tested if the donor Treg in scurfy mice enhanced the cell death of VSAg-reactive T cells. We used the cell surface staining of Annexin V and 7-AAD to determine the early apoptotic cells among VSAg-reactive T cells ($V\beta 3$, 5, 11 or 12^+) or non VSAg-reactive T cells ($V\beta 8^+$). In the untreated scurfy mice, Annexin V staining was comparable among different TCR $V\beta$ expressing cells (Fig 4e). In the treated group, however, more apoptosis was observed among the VSAg-reactive T cells than that among the non-VSAg-reactive T cells. When T cells expressing different Vb were analyzed individually, we observed more cell death was observed in T cells expressing $V\beta 3$ than those expressing $V\beta 11$ or $V\beta 12$. These data demonstrate that Treg preferentially induces the apoptosis of autoreactive T cells in vivo.

4. Higher susceptibility rather than shared specificity explain preferential elimination of VSAg-reactive T cells

We developed an in vitro co-culture system to test if Treg can specifically target VSAg-reactive T cells for their apoptosis. $CD4^+CD25^+$ T cells purified from wild type Balb/C mice were first activated for 72 hours by plate bound anti-CD3 and IL-2. Then, lymph node cells from 8-10 days old scurfy mice were incubated with pre-activated Treg with 1:1 ratio in 37°C . Four hours after the incubation, dead cells were determined by 7-AAD staining. We compared the death of $V\beta 5^+$ and $V\beta 8^+$ CD4 T cells. As shown in Fig. 5a, Treg triggered the death of $V\beta 5^+$ but not $V\beta 8^+$ T cells.

Since negative selection enriched VSAg-reactive T cells among Treg, we tested the possibility that higher ratio of Treg/target among $V\beta 5^+$ T cells may explain its preferential elimination. $V\beta 5^+$ T cells were depleted from the $CD25^+$ CD4 T cells prior to pre-activation and co-culture. As shown in Fig. 5b, $V\beta 5$ -depleted Treg still preferentially killed $V\beta 5^+$ CD4 T cells. To further confirm that the shared specificity between Treg and target cells is not required for Treg-mediated cytolysis, we tested the capacity of TCR $V\beta 8^+$ Treg to induce the death of TCR $V\beta 5^+$ Scurfy T cells. As shown in Figure 5c, $V\beta 8^+$ Treg had the similar efficiency in inducing the death of target cells as the $V\beta 8^-$ Tregs.

The above data indicate that VSAg-reactive T cells are more susceptible to apoptosis. To substantiate these observations, we generated alloreactive T cells by stimulating the B10BR spleen cells with irradiated BALB/c spleen cells. Five days later, we carried out co-culture experiments and evaluated killing of $V\beta 5^+$ and $V\beta 8^+$ T cells. As shown in Fig. 5d, $V\beta 5^+$ T cells were more effectively lysed by alloreactive T cells. Thus, higher susceptibility of VSAg-reactive T cells is not limited to Treg.

Discussion

We used VSAg as the model self-antigen to study the specificity and mechanism of Treg-mediated suppression of autoreactive T cells and showed that Treg preferentially kills autoreactive T cells and that the specificity is based on increased susceptibility of VSAg-reactive T cells to T cell-mediated cytolysis. Our conclusion is based on several lines of evidence.

First, we have demonstrated that despite equal efficiency in clonal deletion, VSAg-reactive T cells have a very different fate in the mice with or without Treg. In mice with a mutation of the *Foxp3* gene, VSAg-reactive T cells expanded in the periphery. In contrast, in mice with normal Treg, the representation of VSAg-reactive T cells was further diminished in the periphery. Interestingly, in the Treg-sufficient WT-mice, the disappearance of the VSAg-reactive T cells occurred despite their preferential proliferation in vivo. However, we did not

observe significantly more apoptotic cells in the VSAg-reactive T cells than in the non-VSAg-reactive T cells in WT (data not shown). We reasoned that in wild type mice, apoptotic cells are quickly removed in vivo, such that in the steady state, apoptotic cells are not visible by Annexin V staining. In the mutant mice, accumulation of autoreactive T cells allowed us to observe the Treg-induced apoptosis of VSAg-reactive T cells. The expansion and disappearance of the VSAg-reactive T cells was demonstrated more than 15 years ago [13]. Our data provided direct evidence that the elimination of the VSAg-reactive T cells in the periphery is dependent on Treg.

Second, we showed that Treg are cytotoxic to VSAg-reactive T cells that are expanded in the absence of Treg both in vivo and in vitro. Thus, when Treg was transferred into the Scurfy host, higher proportion of apoptotic T cells was observed among T cells expressing VSAg-reactive TCR. In addition, co-culture of total activated Treg with the lymph node cells from the Scurfy mice led to preferential killing of VSAg-reactive T cells. These data provide evidence that VSAg-reactive T cells are more susceptible to lysis by T cells, including both Treg and conventional alloreactive T cells. The cellular difference between the autoreactive T cells and other T cells remains to be elucidated. We have not observed any difference in expression of Fas (data not shown). In addition, since the non-VSAg-reactive T cells in the Scurfy mice proliferated equally well, it is clear that proliferation alone does not confer higher susceptibility. Thus, Treg discriminates self-reactive vs nonreactive proliferating T cells in vivo. This feature allows Treg to control autoreactivity while at the same time preserving significant response to foreign antigens, even though the latter is also subject to Treg control [14].

Besides the cytotoxicity of Treg, other mechanisms might also be involved in the elimination of VSAg-reactive T cells by Treg in vivo. Cytokines secreted by Treg, such as IL-10, has been implicated in promoting the activation induced cell death [15]. Apoptosis of autoreactive T cells induced by Treg in theory can also be caused by the interaction between Treg and other components of the immune system, such as the Treg mediated suppression of DC function. In addition, for some VSAg-reactive and non-VSAg-reactive T cells, Treg also repressed proliferation in vivo (Fig. 3c, Fig. 4d). Obviously, these mechanisms are not mutually exclusive.

Molecular mechanisms of Treg mediated cytotoxicity against VSAg reactive T cells is still an open question. It has been suggested that in vitro suppression of T-cell activation by Treg cells was mediated by a perforin-independent, granzyme-B-dependent mechanism [16]. It would be of interest to test whether a similar pathway may also be involved in the killing of VSAg reactive T cells.

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Fig.1. Endogenous VSAg-specific T cells are effectively deleted in the thymus of scurfy mice
Thymocytes from 15 days old BALB/c scurfy mice or their wild type littermates were stained with anti-CD4, anti-CD8 and various anti-TCR V β antibodies, and analyzed by flow cytometry. a. Representative FACS profiles showing the percentage of CD4⁺CD8⁻ or CD4⁻CD8⁺ thymocytes expressing different TCR V β segments. Numbers in each panel represent the percentage of cells expressing the given TCR V β segments. b. Summary of percentage of CD4 SP (upper panel) and CD8 SP (lower panel) thymocytes expressing the corresponding TCR V β segments. Data are a summary of at least 8 mice per group from 5 independent experiments.

Fig.2. Clonal deletion enriches VSAg-reactive regulatory T cells

Thymocytes and splenocytes from 5-6 weeks old wild type Balb/c mice were stained with CD4, CD8, respective TCR Vβ antibodies and CD25 or FoxP3. VSAg-reactive CD4 T cells were defined as T cells expressing at least one of the following Vβ: Vβ3, Vβ5, Vβ11, or Vβ12, and Vβ8⁺ T cells were used as non-VSAg-reactive T cell control. a. Comparison of FoxP3⁺ T cells in VSAg-reactive CD4 T cells and non-VSAg reactive CD4 T cells in the thymus (left panel) and in the spleen (right panel). b. Summary of percentage of FoxP3⁺ (lower panel) T cells in CD4⁺ VSAg-reactive and CD4⁺ VSAg-non-reactive population of the thymus and spleen. c. Representative FACS profiles comparing VSAg-reactive Treg between deleting background (Balb/C) and non-deleting background (B6). Plots were gated on CD4 splenocytes (upper panel) or CD4SP thymocytes (lower panel) and are representative of two independent experiments.

Fig.3. Selective accumulation of VSAg-reactive T cells in the periphery

Splenocytes and lymph node cells from 15-day old scurfy mice or their wild-type littermates were stained by CD4, CD8 and respective TCR V β antibodies and analyzed by flow cytometry. a. Representative profile of TCR V β expression in the spleen. The numbers on each panel represent the percentage of cells expressing the given TCR V β segments. b. Summary of the distributions of various TCR V β in the spleen. Upper panel shows the distribution in CD4⁺ population; lower panel shows those in CD8⁺ population. Number of mice examined, scurfy n=12; wild type n=9. Similar data were observed in the lymph node (data not shown). c. Proliferation of VSAg-reactive T cells in the lymph nodes. Scurfy mice or their wild-type littermates at 15-day of age were pulsed by BrdU (1mg/mouse) for three hours. Lymph node cells were surface-stained with CD4, CD8, and V β 3, V β 5, V β 8, V β 11, or V β 12 antibodies, and then intracellular stained for BrdU⁺ cells. The numbers on each plot represent the percentage of BrdU⁺ cells in the given TCR V β ⁺ CD4 cells. d. Summary of proliferation of VSAg-reactive T cells in the lymph nodes. Data are summary of 4 mice per group from 2 independent experiments. Similar data were observed in the spleen (data not shown). e. Proliferation of VSAg-reactive T cells in 8-day old Scurfy mice. As in c, proliferation of VSAg-reactive (V β 3, V β 5 or V β 11⁺) CD4 T cells and non-VSAg-reactive(V β 8⁺) CD4 T cells were determined by 3 hour BrdU incorporation. CD4 T cells in the spleen were analyzed and data are representative of two independent experiments. Note the dramatic expansion of VSAg reactive T cells in the Scurfy mice, but comparable proliferation between VSAg and non-VSAg reactive T cells.

Fig.4. Regulatory T cells eliminate VSAg-reactive T cells in scurfy mice

10^6 CD4⁺CD25⁺ T cells isolated from wild type BALB/c mice (Thy1.2⁺) were i.p. injected into 2-3-days old scurfy mice. When the mice reached 15-16 days of age, spleen and lymph node cells were analyzed. a. Accumulation of donor T cells in the lymph nodes of scurfy mice. Live cells from spleen and lymph nodes were analyzed by flow cytometry using antibodies against CD4 and Thy1.2. The numbers indicate the percentage of Thy1.2⁺CD4⁺ or Thy1.2⁻CD4⁺ cells in either lymph nodes or spleen. In the lymph node of Scurfy mice, the CD62L expression is down regulated in comparison to those recovered from Wt recipients (lower panels). b. Reduction of VSAg-reactive T cells caused by adoptive transfer of Treg. Data shown are representative profile of treated- and untreated-spleen and lymph node cells. Numbers indicate the percentages of CD4⁺ T cells expressing corresponding TCR Vβ. c. Summary of the alteration of frequencies of the VSAg-reactive T cells in scurfy mice after the Treg transfer. Data are from three Treg treated scurfy mice and four wild type littermates in two independent experiments. d. Proliferation of VSAg-reactive T cells after the neonatal Treg transfer. Mice were pulsed with BrdU as detailed in Figure 3 legends. CD4⁺ cells from the lymph nodes were analyzed by flow cytometry after the staining of BrdU and corresponding TCR Vβ antibodies. e. Apoptosis of VSAg-reactive T cells in the Scurfy mice with or without Treg transfer. CD4⁺ T cells from the lymph nodes expressing the corresponding TCR Vβ chain were stained by Annexin V and 7AAD. Note similar frequencies of apoptotic cells in VSAg reactive T cells as in non-VSAg reactive T cells without the Treg transfer, while much higher apoptosis in TCR Vβ3⁺ cells than Vβ8⁺ cells after the Treg transfer.

Fig. 5. Increased susceptibility of VSAg-reactive T cells to cell-mediated cytotoxicity

a. Representative profiles of Treg-induced apoptosis of VSAg-specific T cells in vitro. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from syngeneic wild-type mice were stimulated with plate bound anti-CD3 and IL-2 for 72 hours. The pre-activated Treg were then mixed with fresh Scurfy lymph node cells at 1:1 ratio for 4 hours. Thy1.1⁺CD4⁺ V β 5⁺(VSAg reactive) or Thy1.1⁺CD4⁺ V β 8⁺(non-VSAg reactive) cells were gated and analyzed as target cells. Death of the target cells was determined by the % of 7-AAD⁺ cells. Upper panels depict the gating strategy and low panels show the 7AAD staining of target cells with different effector cells. b. Treg-mediated cytotoxicity in vitro does not require shared specificity between Treg and target cells. Left panel, V β 5⁻ CD4⁺CD25⁺ T cells was tested for their killing capacities against V β 5⁺ target cells; Right panel, purified V β 8⁺ Treg was tested. Upper panels show the representative profiles of purified effector cells, while the lower panels show the summary of specific lysis induced by different effector cells, as calculated by % of 7-AAD⁺ cells with effector cells minus % of 7-AAD⁺ cells without effector cells. Each sample was in triplicate and data are representative of two independent experiments. c. As in b, except that alloreactive T cells were used as effector cells. The experiments have been repeated twice with similar results. Data in b and c are presented as Mean+SE.