TLR2-Dependent Induction of IL-10 and Foxp3+CD25+CD4+ Regulatory T Cells Prevents Effective Anti-Tumor Immunity Induced by Pam2 Lipopeptides In Vivo

Sayuri Yamazaki1*, Kohei Okada1*, Akira Maruyama1, Misako Matsumoto1, Hideo Yagita2, Tsukasa Seya1*

1 Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Sapporo, Japan, 2 Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Abstract

16 S-[2,3-bis(palmitoyl)propyl]cysteine (Pam2) lipopeptides act as toll-like receptor (TLR)2/6 ligands and activate natural killer (NK) cells and dendritic cells (DCs) to produce inflammatory cytokines and cytotoxic NK activity in vivo. However, in this study, we found that systemic injection of Pam2 lipopeptides was not effective for the suppression of NK-sensitive B16 melanomas in vivo. When we investigated the immune suppressive mechanisms, systemic injection of Pam2 lipopeptides induced IL-10 in a TLR2-dependent manner. The Pam2 lipopeptides increased the frequencies of Foxp3+CD4+ regulatory T (T reg) cells in a TLR2- and IL-10-dependent manner. The T reg cells from Pam2-lipopeptide injected mice maintained suppressor activity. Pam2 lipopeptides, plus the deletion of T reg with an anti-CD25 monoclonal antibody, improved tumor growth compared with Pam2 lipopeptides alone. In conclusion, our data suggested that systemic treatment of Pam2 lipopeptides promoted IL-10 production and T reg function, which suppressed the effective induction of anti-tumor immunity in vivo. It is necessary to develop an adjuvant that does not promote IL-10 and T reg function in vivo for the future establishment of an anti-cancer vaccine.

Citation: Yamazaki S, Okada K, Maruyama A, Matsumoto M, Yagita H, et al. (2011) TLR2-Dependent Induction of IL-10 and Foxp3+CD25+CD4+ Regulatory T Cells Prevents Effective Anti-Tumor Immunity Induced by Pam2 Lipopeptides In Vivo. PLoS ONE 6(4): e18833. doi:10.1371/journal.pone.0018833

Editor: Jacques Zimmer, Centre de Recherche Public de la Sante´ (CRP-Sante´), Luxembourg

Received January 28, 2011; Accepted March 10, 2011; Published April 20, 2011

Copyright: © 2011 Yamazaki et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was funded by Grant for challenging exploratory research, Japan Society for The Promotion of Science Challenging Grand (SY), Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan, the Mochida Memorial Foundation for Medical and Pharmaceutical Research (SY), the Yakult Foundation, and the Waxman Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
* E-mail: seya-tu@pop.med.hokudai.ac.jp (TS); yamazas@med.hokudai.ac.jp (SY)
† These authors contributed equally to this work.

Introduction

Foxp3+CD25+CD4+ regulatory T (T reg) cells constitute about 5–10% of peripheral CD4+ T cells and control immunological self-tolerance and tumor immunity [1,2]. T reg cells directly infiltrate the tumor and suppress effector cells [3–5]. T reg cells are also induced from non-T reg cells in the draining lymph nodes of tumor-bearing mice by transforming growth factor (TGF)-β producing dendritic cells (DCs) [6]. Effective anti-tumor immunity is induced by depletion of T reg cells with anti-CD25 monoclonal antibody (mAb) [7–9], or blockade of T reg function with anti-CTLA-4 mAb [10–12] or anti-GITR mAb [3]. Specific depletion of T reg cells using mice that express diphtheria toxin receptor under the control of the Foxp3 locus induced tumor regression [4,13]. Therefore, strategies are required to abolish the T reg-induced tolerance that suppresses tumor immunity, thereby establishing an effective anti-tumor immune response.

To overcome the immune suppression mediated by T reg cells in cancer, activation of DCs with adjuvants is required [14,15]. Adjuvants are mainly targeted to pattern recognition receptors, such as Toll like receptor (TLR) ligands on DCs. To date, cancer vaccine adjuvants have included various TLR agonists such as TLR3, TLR4, TLR5, TLR7 and TLR9 [16,17]. DCs stimulated by lipopolysaccharide (LPS), a TLR4 agonist, were found to expand functional T reg cells [18,19]. Hence, it is critical to identify the optimal adjuvants that mature DCs but have less potential to expand T reg cells. However, it is unclear how adjuvants differently affect T reg cell survival and function.

The Bacillus Calmette-Guerin-cell wall skeleton (BCG-CWS) is a TLR2 agonist [20] and has been used as an effective adjuvant for cancer for almost 40 years [16,21]. However, its clinical usage is limited since BCG-CWS is a large molecular complex unable to be chemically synthesized with full activity. The anti-cancer activity of BCG-CWS operates partly through TLR2 signal [22–24], hence, we investigated the adjuvant activity of synthetic TLR2/TLR6 ligands derived from Staphylococcus aureus, 16 S-[2,3-bis(palmitoyl)propyl]cysteine (Pam2) lipopeptides. We have previously reported that Pam2 lipopeptides activate DCs and natural killer (NK) cells to produce interferon (IFN)-γ and killer activity in vivo [25] and that local injection of Pam2 lipopeptides with...
RGDS peptides, plus tumor extract, could inhibit tumor growth [26].

Here, we tested if systemic injection of Pam2 lipopeptides in mice could induce an effective anti-tumor immune response. The Pam2 lipopeptides have two palmitoyl-bases attached to different peptide sequences (Fig. 1A) and the peptide portion determines the activity of the TLR2 agonist [25]. We selected the most effective TLR2 activators among the 20 Pam2 lipopeptides [25] and investigated the corresponding anti-tumor response in vivo. In contrast with the in vitro results, systemic injection of Pam2 lipopeptides did not induce regression of NK-sensitive melanomas. Pam2 lipopeptides induced IL-10 and the expansion of T reg cells in vivo in a TLR2-dependent manner. We also found that the depletion of T reg cells by treatment with an anti-CD25 mAb before Pam2 lipopeptide injection, suppressed the tumor growth compared with Pam2-lipopeptide injection alone. These data suggested that systemic injection of Pam2 lipopeptides induced IL-10 and T reg cells, preventing effective tumor immunity in vivo. Our findings demonstrate the importance of studying the effects on T reg cells in vivo prior to the development of adjuvants.

Results

Systemic injection of Pam2 lipopeptides did not induce tumor growth retardation

To examine the anti-tumor effect of the Pam2 lipopeptides in vivo, mice were injected subcutaneously (s.c.) with NK-sensitive B16D8 melanomas into their back [22] and were treated with Pam2 lipopeptides twice a week (Fig. 1B). We selected four kinds of Pam2 lipopeptides, as shown in Fig. 1A, because they strongly activated NK cells through DCs and induced cytotoxic activity in vitro [25]. To our surprise, although the Pam2 lipopeptides activated NK cells in vitro [25], we did not observe effective anti-tumor response in vivo (Fig. 1B). To exclude the possibility that Pam2 lipopeptides were not distributed systemically, we investigated the activation of spleen DCs and NK cells by flow cytometry. The injection of Pam2 lipopeptides up-regulated CD86 and CD40 on splenic DCs (Fig. 1C). Similarly, CD69 was up-regulated in splenic NK cells (Supplemental Fig. S1). Thus, systemic injection of Pam2 lipopeptides was able to activate DCs and NK cells in the spleen, but did not induce effective anti-tumor responses in vivo.

Pam2 lipopeptides induce IL-10 in vitro and in vivo in a TLR2-dependent manner

To investigate why Pam2 lipopeptides could not induce effective anti-tumor responses against NK-sensitive tumors in vivo, we investigated whether Pam2 lipopeptides could activate suppressive factors, such as IL-10 and T reg related molecules. For this experiment, we mainly used a representative Pam2 lipopeptide, Pam2CSK4, since Pam2CSK4 could activate DCs as well as other tested Pam2 lipopeptides in vitro [25].

When the mRNA levels from DCs stimulated with or without Pam2 lipopeptides were analyzed, Pam2 lipopeptides up-regulated retinal dehydrogenase 2 (RALDH2) and IL-10. RALDH2 in DCs activates retinoic acid, which is an important cofactor for TGF-β1 to induce Foxp3 [27,28]. However, Pam2 lipopeptides did not up-regulate the mRNA of TGF-β1 (Fig. 2A).

To confirm whether IL-10 protein is produced from DCs, we stimulated DCs with Pam2 lipopeptides in vitro for 24 hours and the concentration of IL-10 in the supernatants was measured by the ELISA. Bone-marrow derived DCs (BM-DCs) stimulated by Pam2 lipopeptides produced IL-10 (Fig. 2B). IL-10 was also produced by Pam2 lipopeptide-stimulated DCs from the spleen (data not shown). When DCs from TLR2- knockout (TLR2KO) mice were cultured with Pam2 lipopeptides, the production of IL-10 was not detected (Fig. 2B). Hence, IL-10 production was TLR2 dependent. Interestingly, we also found that Pam2 lipopeptides induced IL-10 production from NK cells (Fig. 2C).

To determine whether CD4+ T cells produced IL-10 in the presence of Pam2 lipopeptides, OT II ovalbumin (OVA) transgenic CD4+ T cells were cultured with DCs along with various doses of OVA peptide, with or without Pam2 lipopeptides (Fig. 2D). In the presence of Pam2 lipopeptides, more IL-10 was produced in the culture supernatants when OT II CD4+ T cells were cultured with DCs and antigen (Fig. 2D). Importantly, IL-10 production was increased in an antigen-dose dependent manner (Fig. 2D).

Next, we analyzed the concentration of IL-10 in the serum of Pam2 lipopeptide-treated mice (Fig. 2E). When serum was taken at one day after Pam2CSK4 injection, significant amounts of IL-10 were detected (Fig. 2E), however, Th1, Th2 and Th17 cytokines were not detected (Fig. 2E). IL-10 production in serum was confirmed to be TLR2 dependent because we could not detect IL-10 in Pam2CSK4-treated TLR2KO mice (Fig. 2E). Taken together, these results indicated that Pam2 lipopeptides induce IL-10 both in vitro and in vivo in a TLR2-dependent manner, which might play a role in suppressing tumor immunity induced by Pam2 lipopeptides.

Systemic injection of Pam2 lipopeptides expands T reg cells through the TLR2 dependent production of IL-10

Since Pam2 lipopeptides induce IL-10, we investigated whether systemic injection of Pam2 lipopeptides could affect T reg cell frequencies. IL-10 produced by zymosan plays a role in inducing T reg cells [29]. We found that the frequency of Foxp3+ T reg cells was increased in the spleen and lymph nodes at day 3 after systemic injection of Pam2CSK4 (Fig. 3A). The frequency of T reg cells had returned to normal by day 7 after Pam2CSK4 injection (Supplemental Fig. S2). The increase of T reg cells was dependent on the IL-10 produced by Pam2CSK4, mice were injected with neutralizing anti-IL-10 mAb (JES5-2A5) and Pam2CSK4 (Fig. 3C). Control mice injected with anti-IL-10 mAb alone or untreated mice were not included in this experiment, however, the frequency of Foxp3+ T reg cells in the mice injected with anti-IL-10 Ab alone would be expected be similar to that of naive mice since it is reported that the frequency of Foxp3+ T reg cells is not affected in the spleen of IL-10 [30] or IL-10 receptor β knockout mice [31]. After three days, co-administration of anti-IL-10 mAb blocked the increase of T reg cells after Pam2CSK4 injection (Fig. 3C).

Therefore, Pam2 lipopeptides expand Foxp3+ T reg cells at day 3 after systemic injection in a TLR2- and IL-10 dependent manner.

T reg cells from Pam2 lipopeptide-treated mice have suppressive activity

Next, we investigated the suppressive function of T reg cells in Pam2 lipopeptide-treated mice. We purified CD25+CD4+ T cells from naive mice or Pam2 lipopeptide-treated mice by flow cytometry (Fig. 4A). The frequency of Foxp3+CD4+ T cells in the purified CD25+CD4+ T cells from naive mice or Pam2
lipopeptide-injected mice was always >95%, as shown in Fig. 4A. The purified CD25⁺CD4⁺ T cells were used for the classical in vitro suppression assay [32]. We found that the CD25⁺ T reg cells from Pam2CSK4-treated mice suppressed the proliferation of CD25⁻ CD4⁺ T cells from naïve mice to a similar degree compared with the CD25⁺ T reg from naïve mice (Fig. 4B, C). This indicated that Pam2 lipopeptides maintain T reg cell function in vivo.

Depletion of T reg cells improves the anti-tumor response by systemic injection of Pam2 lipopeptides

To determine whether systemic injection of Pam2 lipopeptides activates the function of T reg cells and suppresses anti-tumor responses against NK-sensitive tumors in vivo, we used an anti-CD25 mAb (PG61) to deplete T reg cells in vivo before challenge with Pam2 lipopeptide and tumor cells [7–9]. Mice were injected with anti-CD25 mAb on day –3 and challenged with B16D8 melanoma cells on day 0, with or without Pam2CSK4 (Fig. 5A). As previously reported, depletion of T reg cells alone induced growth retardation of tumors [7–9]. Tumor growth was slightly promoted by Pam2CSK4 injection alone (Fig. 5A). However, the tumor growth in mice treated with anti-CD25 mAb plus Pam2CSK4 was slower than in mice treated with Pam2CSK4 alone (Fig. 5A, B). These results suggested that the presence of T reg cells suppressed effective anti-tumor responses after systemic injection of Pam2 lipopeptides.

Figure 1. Pam2 lipopeptides do not induce effective anti-tumor immunity. (A) Structures of the Pam2 lipopeptides are shown. (B) Mice were injected with B16D8 melanoma cells (2×10⁵) on back. The mice were injected s.c. into their footpad with the indicated Pam2 lipopeptides (10 nmol) or saline twice a week, as indicated by arrows, starting from day 0. Tumor growth was monitored in a blind manner. A cross indicates the death of one mouse. One of two experiments is shown. (C) Mice were injected with the indicated Pam2 lipopeptides (10 nmol) or saline. After 12–16 hours, spleen DCs were analyzed by flow cytometry. Plots were gated on CD11c⁺ cells. One of two experiments is shown.

doi:10.1371/journal.pone.0018833.g001
Figure 2. Pam2 lipopeptides induce IL-10 and retinal dehydrogenase. (A) Spleen DCs from B6 mice were cultured with or without 100 nM of Pam2CSK4 or Pam2-#12. After four hours, total RNA was prepared and real-time PCR was performed. Expression of each sample was normalized to GAPDH mRNA expression and fold increases of each sample were calculated to the expression levels at 0 hours. One of two experiments is shown. (B) BM-DCs (1 × 10^5) from wild type (WT) or TLR2KO mice were cultured with or without 100 nM of Pam2-#6, Pam2-#12 and Pam2CSK4 for 24 hours. The culture supernatants were measured for IL-10. One of two experiments is shown. (C) NK cells (2 × 10^5) from spleens were cultured with 100 nM of Pam2-#6, Pam2-#12 and Pam2CSK4 for 24 hours. The culture supernatants were measured for IL-10. One of two experiments is shown. (D) OT II CD4^+ T cells (5 × 10^4) were cultured with spleen DCs (5 × 10^5) with or without 100 nM of Pam2CSK4 or Pam2-#12 and the various doses of OVA peptide. After five days, supernatants were measured for IL-10. The means ±/− SDs from two separate experiments is shown. (E) WT or TLR2KO mice were i.p. injected with 10 nmol Pam2CSK4 and next day serum was measured for the indicated cytokine concentrations.

doi:10.1371/journal.pone.0018833.g002
Pam2 Lipopeptides Induce IL-10 and T Reg In Vivo

A

None

Pam2CSK4

LN

10.7

14.6

P<0.05

WT

None

Pam2CSK4

Sp

11.8

18.4

P<0.05

B

None

Pam2CSK4

LN

10.9

10.7

n.s.

TLR2KO

Sp

14.4

13.5

n.s.

C

Pam2CSK4

Pam2CSK4 + anti-IL-10 Ab

LN

13.9

10.2

Sp

Pam2CSK4

Pam2CSK4 + anti-IL-10 Ab

18.3

13.3
Figure 3. Systemic injection of Pam2CSK4 expands Foxp3+ T reg cells in a TLR2- and IL-10-dependent manner. (A) WT mice were i.p. injected with Pam2CSK4 (10 nmol). After three days, spleen (Sp) and lymph node (LN) cells were analyzed for the expression of Foxp3. The plots were gated on CD4+ T cells. One of four experiments is shown for the FACS plots. The image summarizes the results of four separate experiments. P value is derived from the student’s-t test. (B) As in (A), but TLR2KO mice were injected with Pam2CSK4. One of two experiments is shown for the FACS plots. (C) As in (A), but mice were i.p. injected with Pam2CSK4 with or without 200 μg of anti-IL-10 mAb. One of two experiments is shown.

doi:10.1371/journal.pone.0018833.g003

Discussion

Here, we showed that systemic injection of Pam2 lipopeptides did not induce effective tumor immunity presumably because of the induction of IL-10 and T reg cells. To treat cancer, it is necessary to develop new adjuvants to activate immunity in immune-suppressed patients. Adjuvant activity is generally screened by analyzing its effect on effector cells such as NK cells and CD8+ cytotoxic T cells. However, our results indicated that it is also important to investigate the activity of adjuvants on suppressive factors, such as IL-10 and T reg cells, particularly in vivo.

IL-10 is a key cytokine for IL-10 producing Tr1 regulatory T cells [33], and has also been shown to be an important cytokine for Foxp3+ T reg cells. IL-10 production by Foxp3+ T reg cells is required for the prevention of colitis [34,35]. The specific deletion of IL-10 in Foxp3+ T reg cells in mice induces inflammation especially in the intestine, indicating that IL-10 derived from T reg cells plays a critical role in controlling colitis [35]. Furthermore, M. Kronenberg and his colleagues recently found that IL-10 secreted by other cells is needed for T reg cells to sustain expression of Foxp3 and prevent colitis [31]. This indicated that IL-10-enriched environments are preferable for Foxp3+ T reg cells to exert their suppressive function in vivo. Here we have shown that systemic injection of Pam2 lipopeptides induces IL-10-rich environments in vivo, which could play a role in promoting T reg cell function.

Our results showed that TLR2-dependent production of IL-10 plays a role in expanding T reg cells in vivo (Fig. 3). This is consistent with a recent report by B. Pulendran and his colleagues who showed that TLR2 signaling by zymosan induces IL-10 and retinal dehydrogenase in DCs, which are critical for inducing T reg cells [29]. Zymosan binds to TLR2 and dectin-1 [29]. Our data showed that the TLR2 signal induced by Pam2 lipopeptides has a similar effect to the signal induced by zymosan. The TLR2 signal induced by zymosan results in the active suppression of experimental autoimmune encephalomyelitis (EAE) [29]. Furthermore, various TLR signals prevent the development of autoimmune type 1 diabetes in non-obese diabetic mice [36]. Our results showed that systemic injection of Pam2 lipopeptides was ineffective at inducing tumor immunity. However, it is possible that the Pam2 lipopeptides might be useful in inducing tolerance in the case of autoimmune, allergy or transplant rejection.

In addition to the evidence that IL-10 produced in response to the TLR2 signal affects Foxp3+ T reg cell function, the TLR2 signal can also directly act on T reg cells and promotes their survival [37]. Taken all together, although TLR2 activation by Pam2 lipopeptides is able to induce inflammatory cytokines and activate NK cells in vitro [25], the systemic injection of Pam2 lipopeptides as cancer adjuvants is ineffective at abolishing immune suppression. Whereas, the effective cancer adjuvant, BCG-CWS, activates not only TLR2, but also TLR4 and NOD2 receptors [23,30]. TLR2 activation by Pam2 lipopeptides could activate T reg cells in vivo and the T reg cells could suppress NK function and activation [39–41]. Our preliminary experiments showed that T reg cells actually suppress IFN-γ production from NK cells stimulated with DCs plus Pam2CSK4 (S.Y., K.O., T.S., unpublished data). Here we showed that depletion of T reg cells with adjuvant might be one potential strategy to cancel the effect of activating suppressive factors by Pam2 lipopeptides.

We also found that Pam2 lipopeptides induce IL-10 production from NK cells in vivo (Fig. 2C). It has been known for over a decade that NK cells produce IL-10 [42–44]. Recent reports showed that IL-10 produced by NK cells play an important role in controlling T cell responses [45,46] and anti-inflammatory responses [47]. Moreover, IL-10 enhances the killing by NK cells of autologous antigen presenting cells [48,49]. These reports suggested that IL-10-stimulated NK cells could kill autologous macrophages and DCs, which may result in suppressing effective anti-tumor immunity. Therefore, it is possible that systemic injection of Pam2 lipopeptides in our system may induce IL-10 from NK cells and suppress anti-tumor response in vivo.

In contrast to the systemic injection of Pam2 lipopeptides, local injection of Pam2 lipopeptides was effective at suppressing tumor growth when the Pam2 lipopeptide was fused to RGDs-integrin peptides and injected around the tumor with tumor extracts [26]. This was probably effective for a few reasons: 1) the peptide part of the Pam2 lipopeptide was fused with RGDs, which could promote the binding of Pam2 lipopeptides to DCs; 2) local injection of Pam2 lipopeptides around the tumor may be different from systemic injection of Pam2 lipopeptides in terms of inducing IL-10 and T reg cells. Other literature has also indicated that local administration of Pam2 lipopeptides could be effective for cancer [50,51]. The differential effect on inducing IL-10 and T reg cells between local administration and systemic injection of adjuvants should be investigated further in future studies.

The literature on TLR2 signaling and T reg cells is controversial. Some groups reported that T reg cells temporally lost their suppressive capacity in the presence of the TLR2/TLR1 ligand Pam3CSK4, which contains 3-palmitoyl bases [52,53]. However, a recent report from E. Shevach and his colleagues showed that the presence of Pam3CSK4 in the culture actually showed that anti-tumor immunity. Therefore, it is possible that systemic injection of Pam2 lipopeptides in our system may induce IL-10 from NK cells and suppress anti-tumor response in vivo.

To fight to cancer, it is very important to develop an adjuvant to activate immunity. However, it is also crucial to consider the effect of adjuvants on suppressive factors such as IL-10 and T reg cells. The combination of adjuvant and blockade of IL-10 or T reg cell function might prove a successful strategy for improving cancer vaccines.
Materials and Methods

Mice

C57BL6J (B6) mice and CB17SCID mice were obtained from Japan Clea (Tokyo, Japan). TLR2KO mice were provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). OT II OVA CD4 transgenic mice were kindly provided from Dr. Kazuya Iwabuchi (Kitasato University, Kanagawa, Japan). The mice were maintained in the Hokkaido University Animal Facility (Sapporo, Japan) in specific pathogen free condition. All

Figure 4. T reg cells from Pam2CSK4-treated mice maintain suppressive activity. (A) CD25^+CD4^+ T cells purified by flow cytometry were further fixed and stained with Foxp3. FACs plots were gated on CD4^+ T cells. One of three similar experiments is shown. (B) B6 mice were i.p. injected with Pam2CSK4 (10 nmol) on days 0, 3 and 7. On day 14, CD25^+CD4^+ T cells purified as in (A) were used for the suppression assay. CFSE-labeled CD25^+CD4^+ T cells (5 x 10^4) were stimulated with irradiated spleen antigen presenting cells (1 x 10^5) with or without 5% anti-CD3 mAb supernatant. The purified CD25^+CD4^+ T cells from naïve mice or Pam2CSK4-treated mice were added at the indicated ratio. After three days, cells were stained with CD4 and analyzed with CFSE dilution. Dead cells were eliminated by TOPRO-3. One of three similar experiments is shown. (C) As in (B), but the numbers of live CFSE^+CD4^+ T cells per culture were plotted. One of three similar experiments is shown.

doi:10.1371/journal.pone.0018833.g004
experiments used mice that were between 6–12 weeks-of-age at the time of first procedure. All mice were used according to the guidelines of the institutional animal care and use committee of the Hokkaido University, who approved this study as ID number: 08-0243, “Analysis of immune modulation by toll-like receptors”.

Antibodies and reagents

PE-conjugated CD25 (PC61), Alexa-488 conjugated anti-CD25 (7D4), FITC or APC conjugated CD4 (RM4-5), CD11c, NK1.1, purified anti-CD16/CD32 (2.4G2), purified anti-CD3 (2C11), and isotype antibodies were obtained from Biolegend (San Diego, CA, USA). Anti-CD11c, anti-NK and streptavidin microbeads were purchased from Miltenyi Biotec (Gladbach, Germany). Carboxy-fluorescein diacetate succinimidyl ester (CFSE) and TOPRO-3 were from Molecular Probes (Eugene, OR, USA). The anti-mouse Foxp3 (FJK-16s) staining kit was from eBioscience (San Diego, CA, USA). Purified anti-CD25 (PC61) mAb was a gift from Dr. Ralph Steinman (The Rockefeller University, NY, USA) and anti-CD25 hybridoma cells were from Dr. Jun Shimizu (Kyoto University, Kyoto, Japan). Some of the anti-CD25 (PC61) mAb was produced in CB17SCID mice in our animal facility and purified by ammonium sulfate precipitation. Purified anti-IL-10 mAb (JES5-2A5) was prepared as described previously [54]. Pam2CSK4, Pam2CSK2 and MALP2 short lipopeptides were synthesized by Biologica Co. Ltd (Nagoya, Japan). Pam2-6 and Pam2-12 were from Dr. Yukari Fujimoto and Dr. Koichi Fukase (Osaka University, Osaka, Japan).

Cell isolations

CD4+ T cells were first negatively separated by MACS beads from lymph nodes and spleen cell suspensions (>90%) (Miltenyi Biotech) and T reg cells were further purified by a FACS Aria II (BD Bioscience, Franklin Lakes, NJ, USA). Spleen CD11c+ DCs were selected with anti-CD11c beads (Miltenyi Biotech). Bone marrow DCs (BM-DCs) were cultured with GM-CSF as previously described [22]. NK cells were purified from spleen by anti-NK beads (Miltenyi Biotech). To analyze the activation of DCs and NK cells in vivo by Pam2 lipopeptides, 10 nmol of Pam2 lipopeptides was subcutaneously (s.c.) or intraperitoneally (i.p.) and 12–16 hours later, the spleen was analyzed by flow cytometry. Both routes of injection gave similar results.

Measuring cytokine production

DCs or NK cells were stimulated with 100 nM of Pam2 lipopeptides for 24 hours and the supernatants were measured for IL-10 by ELISA (eBiosciences). CD4+ T cells from OT II transgenic mice were cultured with spleen DCs with or without 100 nM of Pam lipopeptides at the various doses of OVA peptide for five days. The supernatants were measured for IL-10 by ELISA. Serum from Pam2 lipopeptides treated mice or control mice were taken one day after i.p. injection and were measured for IL-10, INF-γ, IL-4 and IL-17 by Cytometric Bead Array (BD Bioscience). Analysis with the Cytometric Bead Array was performed according to the manufacturer’s instructions.

Quantitative PCR

Total RNA was isolated with TRIzol (Invitrogen by life technologies, Carlsbad, CA, USA), and reversed transcribed by High Capacity cDNA Transcription Kit (ABI by life technologies, Carlsbad, CA, USA) according to manufacturer instructions. The qPCR was performed with the Step One Real-Time PCR system (ABI). The primers used for real-time PCR have been reported previously [29].

In vivo tumor challenge

Mice were s.c. injected with 2–3×10^5 B16D8 cells into the back. B16D8 melanoma is a NK-sensitive B16 melanoma cell line, which we have previously established [22]. The tumor growth was monitored twice a week. Sometimes mice were pre-treated with

Figure 5. NK-sensitive tumors grow slowly when Pam2CSK4 is injected into T reg-depleted mice. (A) B6 mice were i.p. injected with 500 μg of anti-CD25 mAb (PC61) on day -3. The mice were injected with B16D8 melanoma cells (2×10^6) into their back on day 0. Pam2CSK4 (10 nmol) or PBS was injected twice a week from day 0 to day 14. Tumor growth was monitored twice a week. n = 3 for each group. A cross indicates the death of one mouse. One representative experiments from two similar experiments is shown. (B) As in (A), but the survival curve summarized from two separate experiments is shown. doi:10.1371/journal.pone.0018833.g005
500 μg of anti-CD25 mAb three days before tumor challenge. Then, 10 nmol of Pam2 lipopeptides or control saline was s.c injected into footpad or i.p. injected twice a week. The both routes of injection gave similar results.

In vitro suppression assay using T reg cells

The classical in vitro suppression assay was performed as previously described [32,55]. Briefly, CD25+CD4+ T cells were purified by flow cytometry and used as suppressor cells. CFSE-labeled CD25-CD4+ T cells or CD4+ T cells were stimulated with or without anti-CD3 mAb (2C11) and 1-5×20 Gy irradiated spleen cells. Various numbers of suppressor cells were added to the culture. After three day culture, cells were stained with CD4-PE and dead cells were gated out with TOPRO-3 (Molecular Probes). All cells in each culture were acquired using the FACS calibur (BD Bioscience) to have the cell yield and number of live CFSE+ cells/culture was calculated. Analysis was performed with Flowjo software (TreeStar, USA).

Supporting Information

Figure S1 NK cells up-regulates CD69 after systemic injection of Pam2 lipopeptides. Mice were subcutaneously injected with the indicated Pam2 lipopeptides (10 nmol) or saline.

References

1. Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. Int J Cancer 127: 759-767.
2. Curiel TJ (2008) Regulatory T cells and treatment of cancer. Curr Opin Immunol 20: 241-246.
3. Ko K, Yamanagi S, Nakamura K, Nishioka T, Hirota K, et al. (2003) Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor infiltrating Foxp3+CD25+CD4+ regulatory T cells. J Exp Med 202: 855-891.
4. Li X, Kostareli E, Suhner J, Garbi N, Hammerling GJ (2010) Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. Eur J Immunol 40: 3235-3235.
5. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, et al. (2004) Specific rejection of large tumors. Nature Med 10: 329–335.
6. Ghiringhelli F, Piug PE, Roux S, Parcellier A, Schmitt E, et al. (2005) Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med 202: 919-929.
7. Shimizu J, Yamanagi S, Sakaguchi S (1999) Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 163: 5211-5218.
8. Ohnizu K, Tawara I, Shimizu J, Sakaguchi S, Fujita T, et al. (1999) Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 59: 3129-3135.
9. Teng MW, Swann JB, von Scheidt B, Sharkey J, Zerafa N, et al. (2010) Multiple antitumor mechanisms downstream of prolyl hydroxylase regulatory T-cell depletion. Cancer Res 70: 2665-2674.
10. Sunthuller RP, van Daalvenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, et al. (2001) Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25+ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 194: 823-832.
11. Quezada SA, Peggs KS, Curran MA, Allison JP (2006) CTLA4 blockade and depletion of CD25+ regulatory T cells by anti-CD25 mAb three days before tumor challenge. Nat Immunol 7: 1178–1184.
12. Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. Nature 449: 419-430.
13. Steinman RM, Nussenzweig MC (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proc Natl Acad Sci U S A 99: 351–358.
14. Dubovsky DV, Jr, Reed SG (2010) Adjuvants for cancer vaccines. Semin Immunol 22: 153-161.
15. Seya T, Matsumoto M (2009) The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 58: 1173-1184.
16. Dubensky TW, Jr., Reed SG (2010) Adjuvants for cancer vaccines. Semin Immunol 22: 153-161.
17. Seya T, Matsumoto M (2009) The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 58: 1173-1184.
18. Yamanagi S, Yoda T, Tarbell K, Olson K, Velinzon K, et al. (2003) Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. J Exp Med 198: 235-247.
19. Yamanagi S, Patel M, Harper A, Bonito A, Fukushima H, et al. (2006) Effective expansion of alloantigen-specific Foxp3+ CD25+ CD4+ regulatory T cells by dendritic cells during the mixed leukocyte reaction. Proc Natl Acad Sci U S A 103: 2758-2763.
20. Tsuji S, Matsumoto M, Takeuchi O, Akira S, Azuma I, et al. (2000) Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guérin: involvement of toll-like receptors. Infect Immun 68: 6883–6880.
21. Azuma I, Seya T (2001) Development of immunoadjuvants for immunotherapy of cancer. Int Immunopharmacol 1: 1249–1259.
22. Akaza T, Ehlharra T, Okuno M, Okada Y, Shingai M, et al. (2007) Antitumor NK cell activation mediated by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci U S A 104: 252-257.
23. Uehori J, Matsumoto M, Tsurugi T, Akaza T, Takeuchi O, et al. (2003) Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by Mycobacterium bovis bacillus Calmette-Guérin pepidoglycan. Infect Immun 71: 4238-4249.
24. Murata M (2006) Activation of Toll-like receptor 2 by a novel preparation of cell wall skeleton from Mycobacterium bovis BCG Tokyo (SMP-165) sufficiently enhances immune response against tumors. Cancer Sci 99: 1433–1440.
25. Azuma M, Sawahata R, Ako Y, Ehlharra T, Yamanagi S, et al. (2010) The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation. PLoS One 5: e12550.
26. Akaza T, Inoue N, Shime H, Kodama K, Matsumoto M, et al. (2010) Adjuvant engineering for cancer immunotherapy: Development of a synthetic TLR2 ligand with increased cell adhesion. Cancer Sci 101: 1596-1593.
27. Sun CM, Hall JA, Blank RB, Bouladou N, Oudka M, et al. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3+regulatory T cells via retinoic acid. J Exp Med 204: 1775–1785.
28. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, et al. (2007) A functionally specialized population of murine CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 204: 1757-1764.
29. Manicasanny S, Ravidran D, Deng J, Oluoch H, Denning TL, et al. (2009) Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. Nat Med 15: 401–409.
30. Collison LW, Pillai MR, Charuvardi V, Vignali DA (2009) Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. J Immunol 182: 6121-6128.
31. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, et al. (2009) Interleukin-10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 10: 1178–1184.
32. Yamanagi S, Bonito AJ, Spieks R, Bhodapakar M, Inaba K, et al. (2007) Dendritic cells are specialized accessory cells along with TGF-β for the...
differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3- precursors. Blood 110: 4293–4302.

33. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, et al. (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 212: 20–50.

34. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 190: 995–1004.

35. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, et al. (2008) Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28: 546–558.

36. Aumeunier A, Grela F, Ramadan A, Pham Van L, Bardel E, et al. (2010) Systemic Toll-like receptor stimulation suppresses experimental allergic asthma and autoimmune diabetes in NOD mice. PLoS One 5: e11484.

37. Chen Q, Davidson TS, Huter EN, Shevach EM (2009) Engagement of TLR2 does not reverse the suppressor function of mouse regulatory T cells, but promotes their survival. J Immunol 183: 4436–4466.

38. Brooks MN, Rajaram MV, Azad AK, Amer AO, Valdivia-Arenas MA, et al. (2010) NOD2 controls the nature of the inflammatory response and subsequent fate of Mycobacterium tuberculosis and M. bovis BCG in human macrophages. Cell Microbiol 13: 402–418.

39. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, et al. (2005) CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med 202: 1073–1083.

40. Giroux M, Yurchenko E, St-Pierre J, Piccirillo CA, Perreault C (2007) T regulatory cells control numbers of NK cells and CD8alpha immature dendritic cells in the lymph node paracortex. J Immunol 179: 4492–4502.

41. Terme M, Chaput N, Combadiere B, Ma A, Ohteki T, et al. (2008) Regulatory T cells control dendritic cell/NK cell crosstalk in lymph nodes at the steady state by inhibiting CD4+ self-reactive T cells. J Immunol 180: 4679–4686.

42. Mehta A, Donnelly RP, Wong S, Kanegane H, Gerreyn A, et al. (1998) Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. J Immunol 160: 2637–2644.

43. Brady J, Hayakawa Y, Snyth MJ, Nutt SL (2004) IL-21 induces the functional maturation of murine NK cells. J Immunol 172: 2048–2058.

44. Maroof A, Beattie L, Zubairi S, Svensson M, Stager S, et al. (2008) Posttranscriptional regulation of IL10 gene expression allows natural killer cells to express immunoregulatory function. Immunity 29: 295–305.

45. Deniz G, Eren G, Kurucuoezer UC, Kocazic D, Karagaingnids G, et al. (2008) Regulatory NK cells suppress antigen-specific T cell responses. J Immunol 180: 850–857.

46. Lee SH, Kim KS, Fodil-Corum N, Vidal SM, Biron CA (2009) Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD107T cell regulation during viral infection. J Exp Med 206: 2235–2245.

47. Perona-Wright G, Mohrs K, Szaba FM, Kummer LW, Madan R, et al. (2009) Systemic but not local infections elicit immunosuppressive IL-10 production by natural killer cells. Cell Host Microbe 6: 503–512.

48. Schulz U, Kreutz M, Multhoff G, Stueber B, Kohler M, et al. (2010) Interleukin-10 promotes NK cell killing of autologous macrophages by stimulating expression of NGK2D ligands. Scand J Immunol 72: 319–331.

49. Alter G, Kavanagh D, Rihan S, Lopez R, Brooks D, et al. (2010) IL-10 induces aberrant deletion of dendritic cells by natural killer cells in the context of HIV infection. J Clin Invest 120: 1905–1913.

50. Shingu K, Kruschinski C, Luhmann A, Grote K, Tschernig T, et al. (2003) Intratracheal macrophage-activating lipopeptide-2 reduces metastasis in the rat lung. Ann J Respir Cell Mol Biol 28: 316–321.

51. Schmidt J, Welsch T, Jager D, Mullhardt PF, Buchler MW, et al. (2007) Intratracheal injection of the toll-like receptor-2 agonist ‘macrophage-activating lipopeptide-2’ in patients with pancreatic carcinoma: a phase I/II trial. Br J Cancer 97: 598–604.

52. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LV, et al. (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 116: 493–494.

53. Liu H, Kominos-Kousa M, Xu D, Liew FY (2006) Toll-like receptor 2 signaling modulates the functions of CD14+ CD145+ regulatory T cells. Proc Natl Acad Sci U S A 103: 7048–7053.

54. Aramaki O, Inoue F, Takayama T, Shimazu M, Kitajima M, et al. (2005) Interleukin-10 but not transforming growth factor-beta is essential for generation and suppressor function of regulatory cells induced by intratracheal delivery of allogeneic. Transplantation 79: 568–576.

55. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, et al. (2008) CD8+ CD125+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. J Immunol 181: 6923–6933.