ing of RIG-I or DDX60 to dsRNA. Because both RIG-I and DDX60 can bind to dsRNA, the data do not imply an interaction between RIG-I and DDX60. Interestingly, coexpression of both proteins increased the levels of proteins recovered from the cell lysate (Fig. 10).

**DDX60 is important for expression of type I IFN and IFN-inducible genes during viral infection.** We next tested whether DDX60 is involved in cytokine expression during viral infection. RIG-I recognizes VSV and SeV, and MDA5 recognizes PV (3, 21). Interestingly, knockdown of DDX60 reduced IFN-β expression in HeLa cells after VSV, PV, and SeV infection. IFIT-1 and IP10 expression after VSV and SeV infection was also reduced by DDX60 knockdown (Fig. 11). Knockdown of DDX60 caused a marginal effect on expression of IFIT1 and IP10 in response to PV infection. Some of the IFN-α gene expression induced by other sensor molecules might have been responsible for the difference. Unlike viral infection, knockdown of DDX60 did not reduce expression of IFIT1 after IFN-β stimulation (Fig. 12A). Reduction of type I IFN expression was also observed after VSV infection in HEK293 cells (Fig. 12B and C). In addition, DDX60 knockdown reduced IRF-3 dimerization after VSV infection (Fig. 12D). These data indicate that DDX60 is required for RIG-I- and MDA5-dependent type I IFN and IFN-inducible gene expression during viral infection. We also observed that suppression of CPE and viral titers in culture medium induced by DDX60 overexpression can be reduced by IPS-1 knockdown (Fig. 6I and J), confirming that the antiviral activity of DDX60 is dependent on the presence of RLRs. Because the DDX60 helicase domain was found to bind to dsDNA, we examined whether DDX60 is involved in type I IFN expression after infection with HSV-1, a DNA virus. In this case, knockdown of DDX60 reduced expression of IFN-β and IP10 after HSV-1 infection (Fig. 12E and F).

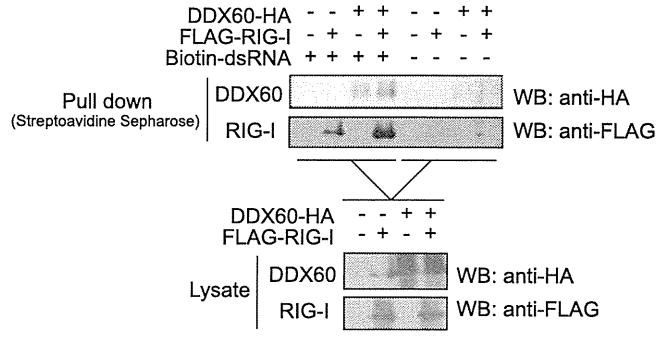


FIG. 10. DDX60 increases the association of RIG-I to short synthetic dsRNA. DDX60- and RIG-I-expressing vectors were transfected into HEK293FT cells. At 24 h later, cell lysate was prepared. The lysate was incubated with or without biotin-conjugated dsRNA, and the dsRNA was recovered using streptavidin Sepharose beads. The recovered fraction was analyzed by Western blotting.

## DISCUSSION

Here, we report that DDX60 is a novel antiviral factor in human cells. The amino acid sequence of DDX60 is similar to that of Ski2 homologs, which are cofactors of the RNA exosome. DDX60 interacts with core components of the RNA exosome. After viral infection, the DDX60 protein binds to endogenous RIG-I protein and is involved in RIG-I-dependent pathway. The protein also binds to MDA5 and LGP2. The DDX60 helicase domain binds to viral RNA and DNA, and coexpression of RIG-I with DDX60 increases the binding of RIG-I and DDX60 to dsRNA. Knockdown of DDX60 reduces expression of type I IFN and IFN-inducible genes after VSV, PV, SeV, and HSV-1 infections. Therefore, we concluded that DDX60 is a novel antiviral helicase involved in RLR-dependent pathways.

Schröder et al. and Soulat et al. first reported that the non-RLR helicase DDX3 plays pivotal roles in RLR-dependent pathways (41, 43). DDX3 is ubiquitously expressed in a variety of cells and exerts its positive effect as a part of TBK-1- and/or IKK-ε-containing complexes that activate IRF-3 (41). DDX3 also binds to RIG-I and IPS-1 and promotes the activation of those proteins (28, 32). Our study showed that another non-RLR helicase, DDX60, is also involved in RLR-dependent pathways. Thus, our reports and previous studies demonstrate the important roles of non-RLR helicases in RLR-mediated signaling and antiviral response.

Because DDX60 protein does not contain CARDs, which are required for the interaction with IPS-1, it seems unlikely that DDX60 directly activates IPS-1 without RLRs. The results of knockdown studies suggest that DDX60 is an upstream factor of IPS-1, and the immunoprecipitation assay results suggest that DDX60 binds to the RLR upstream factors. On the basis of these findings, we expected that DDX60 would

dsRNA. After 24 h, cell lysates were prepared and luciferase activity was measured. (E and F) siRNA for DDX60 or control siRNA was transfected into HEK293 cells. The cells were left unstimulated or stimulated with poly(I · C), and expression of IFN-β and DDX60 mRNA was measured by RT-qPCR. Expression values were normalized using GAPDH. (G) siRNA for DDX60 or the control was transfected into HEK293 cells together with DDX60-expressing vector. The DDX60 protein was observed by Western blotting. (H) Vectors expressing TICAM-1 and/or DDX60 were transfected into HEK293 cells together with the p125luc reporter and *Renilla* luciferase plasmids. After 24 h, the cell lysates were prepared and luciferase activities were measured. (I) Vectors expressing TLR3 and/or DDX60 were transfected into HEK293 cells together with the p125luc reporter and *Renilla* luciferase plasmids. After 24 h, the cells were left unstimulated or stimulated with poly(I · C) for 4 h, the cell lysates were prepared, and luciferase activity was measured. (J and K) HeLa cells expressing shRNA for DDX60 (J) or EXOSC4 (K) were stimulated with 50 μg/ml of poly(I · C) (no transfection) (J) or dsRNA (transfection) (K). RT-qPCR was performed to measure IFN-β mRNA expression. (L) HeLa cells expressing shRNA for GFP, EXOSC4, or EXOSC5 were infected with VSV at an MOI of 1. Levels of induction of IFN-β mRNA were calculated as described for panel J. (M and N) Empty or IPS-1-expressing vector (M) and RIG-I CARD-, MDA5-, or TBK1-expressing vector (N) were transfected into control or DDX60 knockdown HEK293 cells together with p125luc reporter and *Renilla* luciferase plasmids. After 24 h, cell lysates were prepared and luciferase activity was measured. (O) shRNA for DDX60 did not inhibit the signaling from TLR3 (H to J). Although DDX60 promotes RLR-dependent signaling (A to E), shRNA for DDX60 did not reduce the signaling induced by RIG-I CARD, MDA5, IPS-1, or TBK1 overexpression (M and N). These data suggest that shRNA suppresses signaling upstream of RIG-I and MDA5.

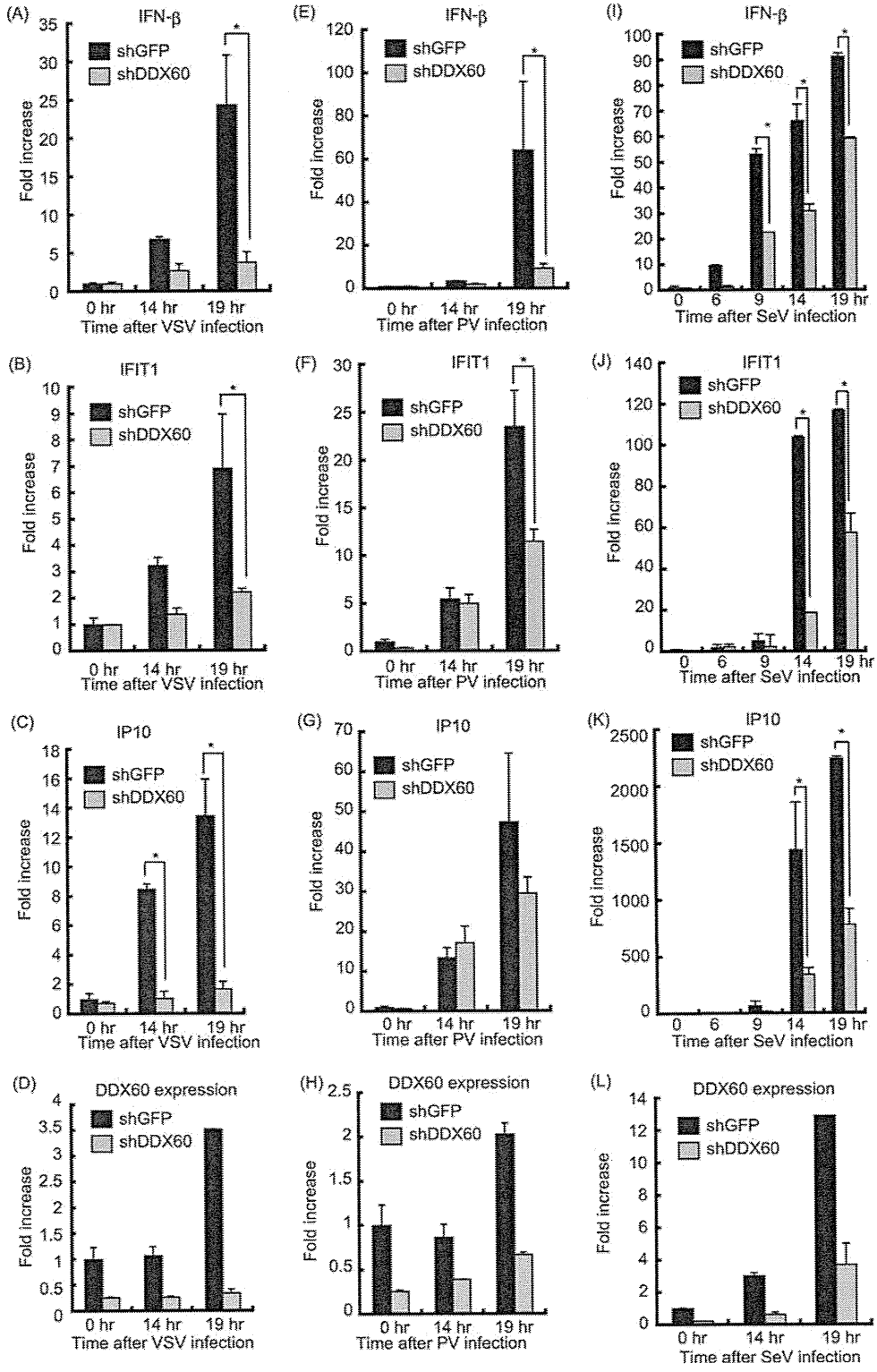


FIG. 11. Knockdown of DDX60 decreases expression of type I IFN during viral infection in HeLa cells. (A to L) Control cells or HeLa cells from a stable cell line expressing shRNA for DDX60 were infected with VSV (A to D), PV (E to H), or SeV (I to L). Total RNA was extracted at the indicated times. RT-qPCR was performed to measure expression of IFN-β (A, E, and I), IFIT1 (B, F, and J), IP10 (C, G, and K), and DDX60 (D, H, and L). The expression level of each sample was normalized to GAPDH expression.

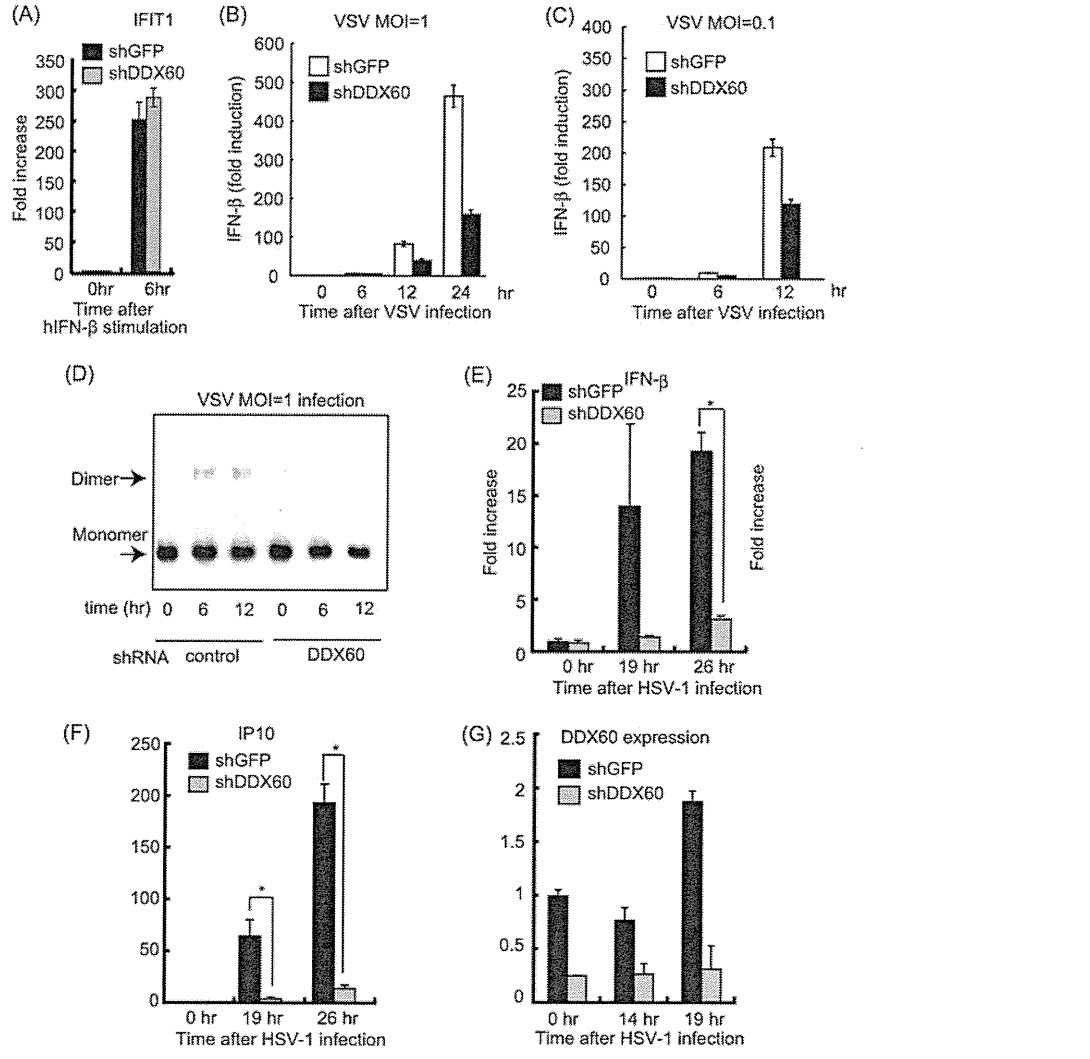


FIG. 12. Effects of DDX60 knockdown on antiviral responses. (A to C) Control or DDX60 knockdown cells were stimulated with human IFN-β (A) or infected with VSV at an MOI of 1 (B) or 0.1 (C), and expression of IFIT1 (A) and IFN-β (B and C) mRNA was examined by RT-qPCR. (D) Control or DDX60 knockdown HEK293 cells were infected with VSV at an MOI of 1, and cell lysates were prepared at the indicated times. Lysates were analyzed by native PAGE, and Western blot analysis was performed with anti-IRF-3 antibody. (E to G) Control or DDX60 knockdown HeLa cells were infected with HSV-1, and total RNA was extracted at the indicated times. RT-qPCR was performed to examine expression of IFN-β (E), IP10 (F), and DDX60 (G).

bind to viral RNA. The gel shift assay showed that DDX60 helicase can bind to dsRNA, and the pulldown assay showed that coexpression of DDX60 with RIG-I increased the binding of RIG-I to dsRNA. Thus, we speculate that DDX60 binds to viral dsRNA and associates with RLRs during viral infection. Further study is required to reveal the precise molecular mechanism by which DDX60 activates the RLR-dependent pathway.

The DDX60 helicase domain binds to dsDNA *in vitro*, and DDX60 was required for type I IFN expression during infection with HSV-1, a DNA virus. In human cells, RIG-I is involved in the pathway activated by cytoplasmic B-DNA or DNA virus infection (1, 6, 7). AT-rich dsDNA is transcribed by RNA polymerase III, and these transcripts are recognized by RIG-I (1, 6). In contrast, Choi et al. reported that the RIG-I protein associates with B-DNA and activates the signaling (7). Previously, Takahasi et al. reported that purified RIG-I protein does not itself bind to dsDNA (46). Therefore, RIG-I seems to associate with B-DNA via another protein that directly binds to dsDNA. HSV-1 was reported to produce considerable amounts of dsRNA and to activate RIG-I and MDA5 (25, 34, 52). Thus, we do not exclude the possibility that DDX60 is involved in recognition not of dsDNA but of dsRNA derived from HSV-1. Although RIG-I and MDA5 are involved in the signaling induced by cytoplasmic B-DNA, IPS-1 is dispensable for the signaling (23). Further study is required to reveal the molecular mechanism by which DDX60 plays an important role in the signaling induced by cytoplasmic B-DNA.

In addition to the *DDX60* gene, the human genome includes the closely related *DDX60L* gene, which is located 5′ upstream of *DDX60* on chromosome IV. However, the mouse genome encodes only one DDX60 protein. Our phylogenetic analysis indicated that the mouse gene is a shared ancestor of human *DDX60* and *DDX60L* genes, and a pilot study has revealed that

DDX60L is expressed after viral infection (unpublished results). Therefore, DDX60L is also expected to be an antiviral protein. Considering that knockdown of DDX60 severely reduced type I IFN expression after viral infection, there seem to be functional differences between DDX60 and 60L. Further study is required to reveal the functional differences between DDX60 and 60L.

The amino acid sequence of DDX60 is weakly similar to those of the exosome cofactors SKIV2L and SKIV2L2. In immunoprecipitation experiments, DDX60 protein was found to coimmunoprecipitate with core components of the RNA exosome. However, we could not determine the physiological role of the interaction between DDX60 and RNA exosome by knockdown analysis. We examined whether or not knockdown of DDX60 and the RNA exosome delays degradation of viral genomic RNA. We found that knockdown of DDX60 or RNA exosome components does not substantially delay degradation of transfected viral RNA (unpublished results). This observation is consistent with our results showing that knockdown of the RNA exosome does not increase viral titers. However, we do not exclude the possibility that the RNA exosome is involved in antiviral responses. There exist several antiviral nucleases, such as RNase L and ISG20. Thus, it is possible that those antiviral nucleases compensate for the defect of the RNA exosome. This possibility is not surprising, because there are several redundant pathways in the innate immune system. For example, poly(I · C) is a ligand common to TLR3 and MDA5; thus, not single but double knockout is required to abolish poly(I · C)-dependent NK cell cytotoxicity (27). Type I IFNs are produced from various kinds of cells, such as fibroblasts, dendritic cells, and macrophages; thus, we do not exclude the possibility that the RNA exosome performs some roles in DDX60 antiviral activity in other cells. The RNA exosome is required to maintain the integrity of host RNA and to disrupt RNA that lacks a 5′ catabolite gene activator protein (CAP) or 3′ poly(A) tail. Thus, it is also possible that DDX60 is involved in host RNA integrity. Further study is required to reveal the physiological role of the association of DDX60 with the RNA exosome.

In single-cell organisms such as budding yeast (*S. cerevisiae*), Ski2 plays a major role in the antiviral response to dsRNA virus. DDX60, a homolog of Ski2, is conserved among eukaryotes. For example, DDX60 is also encoded by the *Caenorhabditis elegans* genome. In a pilot study, we found nematode DDX60 expression to be increased after viral infection (unpublished results), leading us to predict that DDX60 possesses antiviral activity in this species as well. Phylogenetic tree analysis has shown that antiviral helicases such as DDX60, RLRs, and Dicer are clustered into one node. Considering that budding yeast uses Ski2 helicase for its antiviral activity, Ski2-DDX60 protein might represent the most primitive antiviral helicase, which has since diverged into several distinct but similar proteins, such as Dicer and RLRs.

## ACKNOWLEDGMENTS

This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan, the Ministry of Health, Labor, and Welfare of Japan, Mitsubushi Foundation, and Akiyama Foundation.

We thank K. Matsumoto, H. Saito (National Research Institute for Child Health and Development), and H. Takaki for microarray data.

## REFERENCES

1. **Ablasser, A., et al.** 2009. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat. Immunol. **10:**1065–1072.
2. **Akazawa, T., et al.** 2007. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc. Natl. Acad. Sci. U. S. A. **104:**252–257.
3. **Barral, P. M., et al.** 2007. MDA-5 is cleaved in poliovirus-infected cells. J. Virol. **81:**3677–3684.
4. **Brouwer, R., et al.** 2001. Three novel components of the human exosome. J. Biol. Chem. **276:**6177–6184.
5. **Chen, G., X. Guo, F. Lv, Y. Xu, and G. Gao.** 2008. p72 DEAD box RNA helicase is required for optimal function of the zinc-finger antiviral protein. Proc. Natl. Acad. Sci. U. S. A. **105:**4352–4357.
6. **Chiu, Y. H., J. B. Macmillan, and Z. J. Chen.** 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell **138:**576–591.
7. **Choi, M. K., et al.** 2009. A selective contribution of the RIG-I-like receptor pathway to type I interferon responses activated by cytosolic DNA. Proc. Natl. Acad. Sci. U. S. A. **106:**17870–17875.
8. **Cui, S., et al.** 2008. The C-terminal regulatory domain is the RNA 5′-triphosphate sensor of RIG-I. Mol. Cell **29:**169–179.
9. **Dangel, A. W., L. Shen, A. R. Mendoza, L. C. Wu, and C. Y. Yu.** 1995. Human helicase gene SKI2W in the HLA class III region exhibits striking structural similarities to the yeast antiviral gene SKI2 and to the human gene KIAA0052: emergence of a new gene family. Nucleic Acids Res. **23:**2120–2126.
10. **Ebihara, T., et al.** 2010. Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J. Exp. Med. **207:**2675–2687.
11. **Gack, M. U., et al.** 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature **446:**916–920.
12. **Galiana-Arnoux, D., C. Dostert, A. Schneemann, J. A. Hoffmann, and J. L. Imler.** 2006. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nat. Immunol. **7:**590–597.
13. **Gao, D., et al.** 2009. REUL is a novel E3 ubiquitin ligase and stimulator of retinoic-acid-inducible gene-I. PLoS One **4:**e5760.
14. **Guo, X., J. Ma, J. Sun, and G. Gao.** 2007. The zinc-finger antiviral protein recruits the RNA processing exosome to degrade the target mRNA. Proc. Natl. Acad. Sci. U. S. A. **104:**151–156.
15. **Hayakawa, S., et al.** 2011. ZAPS is a potent stimulator of signaling mediated by the RNA helicase RIG-I during antiviral responses. Nat. Immunol. **12:**37–44.
16. **Hornung, V., et al.** 2006. 5′-Triphosphate RNA is the ligand for RIG-I. Science **314:**994–997.
17. **Houseley, J., J. LaCava, and D. Tollervey.** 2006. RNA-quality control by the exosome. Nat. Rev. Mol. Cell Biol. **7:**529–539.
18. **Houseley, J., and D. Tollervey.** 2009. The many pathways of RNA degradation. Cell **136:**763–776.
19. **Kato, H., et al.** 2005. Cell type-specific involvement of RIG-I in antiviral response. Immunity **23:**19–28.
20. **Kato, H., et al.** 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J. Exp. Med. **205:**1601–1610.
21. **Kato, H., et al.** 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature **441:**101–105.
22. **Kawai, T., et al.** 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. **6:**981–988.
23. **Kumar, H., et al.** 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. J. Exp. Med. **203:**1795–1803.
24. **Li, X., et al.** 2009. The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA. J. Biol. Chem. **284:**13881–13891.
25. **Melchjorsen, J., et al.** 2010. Early innate recognition of herpes simplex virus in human primary macrophages is mediated via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent pathways. J. Virol. **84:**11350–11358.
26. **Meylan, E., et al.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437:**1167–1172.
27. **Miyake, T., et al.** 2009. Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. J. Immunol. **183:**2522–2528.
28. **Oshiumi, H., et al.** 2010. Hepatitis C virus core protein abrogates the DDX3 function that enhances IPS-1-mediated IFN-beta induction. PLoS One **5:**e14258.
29. **Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya.** 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. **4:**161–167.
30. **Oshiumi, H., M. Matsumoto, S. Hatakeyama, and T. Seya.** 2009. Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. J. Biol. Chem. **284:**807–817.

31. **Oshiumi, H., et al.** 2010. The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell Host Microbe **8:**496–509.
32. **Oshiumi, H., K. Sakai, M. Matsumoto, and T. Seya.** 1 February 2010, posting date. DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-beta inducing potential. Eur. J. Immunol. doi:10.1002/eji.200940203.
33. **Pippig, D. A., et al.** 2009. The regulatory domain of the RIG-I family ATPase LGP2 senses double-stranded RNA. Nucleic Acids Res. **37:**2014–2025.
34. **Rasmussen, S. B., et al.** 2009. Herpes simplex virus infection is sensed by both Toll-like receptors and retinoic acid-inducible gene-like receptors, which synergize to induce type I interferon production. J. Gen. Virol. **90:**74–78.
35. **Rehwinkel, J., et al.** 2010. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell **140:**397–408.
36. **Saito, T., et al.** 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc. Natl. Acad. Sci. U. S. A. **104:**582–587.
37. **Saito, T., D. M. Owen, F. Jiang, J. Marcotrigiano, and M. Gale, Jr.** 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature **454:**523–527.
38. **Satoh, T., et al.** 26 January 2010, posting date. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.0912986107.
39. **Schlee, M., et al.** 2009. Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity **31:**25–34.
40. **Schmidt, A., et al.** 2009. 5′-Triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. Proc. Natl. Acad. Sci. U. S. A. **106:**12067–12072.
41. **Schröder, M., M. Baran, and A. G. Bowie.** 2008. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. EMBO J. **27:**2147–2157.
42. **Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell **122:**669–682.
43. **Soulat, D., et al.** 2008. The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response. EMBO J. **27:**2135–2146.
44. **Sun, Q., et al.** 2006. The specific and essential role of MAVS in antiviral innate immune responses. Immunity **24:**633–642.
45. **Takahasi, K., et al.** 2009. Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. J. Biol. Chem. **284:**17465–17474.
46. **Takahasi, K., et al.** 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. Mol. Cell **29:**428–440.
47. **van Dijk, E. L., G. Schilders, and G. J. Pruijn.** 2007. Human cell growth requires a functional cytoplasmic exosome, which is involved in various mRNA decay pathways. RNA **13:**1027–1035.
48. **van Rij, R. P., et al.** 2006. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev. **20:**2985–2995.
49. **Venkataraman, T., et al.** 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. J. Immunol. **178:**6444–6455.
50. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
51. **Wang, X. H., et al.** 2006. RNA interference directs innate immunity against viruses in adult Drosophila. Science **312:**452–454.
52. **Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan.** 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J. Virol. **80:**5059–5064.
53. **Wickner, R. B.** 1996. Double-stranded RNA viruses of Saccharomyces cerevisiae. Microbiol. Rev. **60:**250–265.
54. **Widner, W. R., and R. B. Wickner.** 1993. Evidence that the SKI antiviral system of Saccharomyces cerevisiae acts by blocking expression of viral mRNA. Mol. Cell. Biol. **13:**4331–4341.
55. **Xu, L. G., et al.** 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol. Cell **19:**727–740.
56. **Yanai, H., et al.** 2009. HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature **462:**99–103.
57. **Yoneyama, M., and T. Fujita.** 2007. RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. Cytokine Growth Factor Rev. **18:**545–551.
58. **Yoneyama, M., et al.** 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. **175:**2851–2858.
59. **Yoneyama, M., et al.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. **5:**730–737.

Microbiology and Immunology

*Microbiol Immunol* 2011; **55**: 373–377
doi:10.1111/j.1348-0421.2011.00319.x

NOTE

# Development of monoclonal antibodies that specifically interact with necrotic lymphoma cells

Kentaro Wakasa[1,2], Hiroaki Shime[1,3], Mitsue Kurita-Taniguchi[3], Misako Matsumoto[1,3], Masahiro Imamura[2], and Tsukasa Seya[1,3]

[1]Department of Microbiology and Immunology, and [2]Department of Hematology, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, and [3]Department of Immunology, Osaka Medical Center for Cancer, Higashinari-ku, Osaka 537-8151, Japan

## ABSTRACT

**The immune system has evolved mechanisms to sense not only microbes, but also necrotic cells. The pattern-recognition receptors in macrophages/dendritic cells that stimulate the acquired immune system are closely associated with danger signaling. In this study monoclonal antibodies (mAbs) that specifically interact with necrotic cells were developed. One IgG1 and two IgM mAbs were established, and they recognized a 80 kDa protein expressed in necrotic, but not live or apoptotic, cells. These mAbs, which serve as a probe for necrosis, facilitate analyses of the role of the immune complex that consists of necrotic cells and Ab and contributes to the formation of the inflammatory milieu induced by necrotic cell death.**

**Key words** inflammation, monoclonal antibody, necrotic cell death, pattern-recognition receptors.

Necrotic cell death is a common event during inflammatory responses to cancer and microbial infections. This type of cell death clearly differs from apoptosis in its effects on tissue repair and induction of immunological responses (1, 2). Recent progress on the study of innate immunity suggests that necrotic cells liberate a number of proinflammatory agents, including HSPs, high mobility group protein B1, uric acid, galectins and S100 proteins (1–6). Once liberated from necrotic cells, these intracellular proteins contribute to the formation of an inflammatory nest and create a unique tissue microenvironment (1, 2). Furthermore, the complement system is activated around dead cells and enhances inflammation. These events are followed by the recruitment of immune-related cells to the focal nest, and this modifies the inflammatory milieu. Finally, the affected tissue is cleared away and regeneration occurs. Necrosis is a major pathway for initiating productive inflammation.

The necrotic cell death that frequently occurs in malignant tumors is followed by periodical degradation, commonly referred to as chronic or smoldering inflammation. So-called danger signals occur secondary to cell death-mediated events. These signals include liberation of proinflammatory mediators, which are crucial for progression and metastasis of tumor and for evoking tumor immunity (7). Antibodies tend to be generated against necrotic material, presumably with neo-antigen (8). Phagocytic uptake of tumor antigens and inflammatory factors named DAMPs by dendritic cells or macrophages is believed to be important in provoking antitumor responses (1, 2). Surprisingly, tumor-infiltrating macrophages can release DAMPs to the local environment and help the tumor form an inflammatory nest (9). Auto- and/or neo-antigens may appear during the process of formation of the necrotic cell microenvironment. However, the lack of an appropriate system for testing the role of Abs in necrotic conditions

The first two authors contributed equally to this work.

**Correspondence**
Tsukasa Seya, Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo, 060-8638, Japan. Tel: 81 11 706 5073; fax: 81 11 706 7866; email: seya-tu@pop.med.hokudai.ac.jp

Received 5 January 2011; revised 26 January 2011; accepted 27 January 2011.

**List of Abbreviations:** Ab, antibody;DAMP, damage-associated molecular patterns; HMGB, high-mobility group box; HSP, heat shock protein; mAb, monoclonal antibody; PI, propidium iodide; PRR, pattern-recognition receptor; TLR, Toll-like receptor.

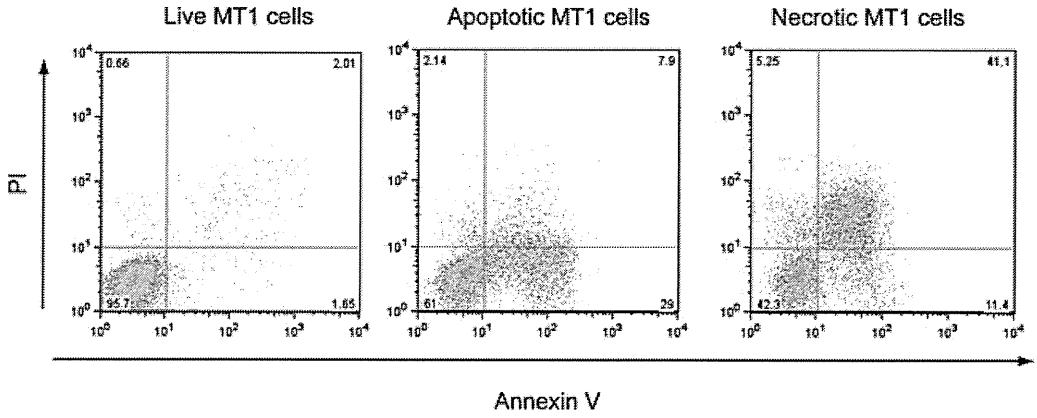


**Fig. 1. Specific induction of apoptosis and necrosis in MT-1 cells.** MT-1 cells ($10^6$/well) were incubated with medium only (left panel), 50 ng/mL of anti-Fas Ab for 4 hr (center panel) or anti-Fas Ab for 15 hr (right panel). The cells were stained with fluorescein isothiocyanate -labeled Ab against Annexin V (X axis) and PI (Y axis) and analyzed by flow cytometry. Apoptotic (annexin V-positive and PI-negative) and secondary necrotic (annexin V-positive and PI-positive) cells were detected separately.

is a deterrent to analyzing the events leading to chronic inflammation involving tumor and immune cells. Here we describe the development of mAbs against a necrotic human lymphoma cell line, MT1. Since these mAbs exclusively gain access to the antigens of necrotic cells without cross-reacting with apoptotic or live cells, they could be used to monitor the events that occur as a result of necrotic cell-specific sensitization by Abs. Such mAbs could be a useful tool for facilitating analysis of the effects of Abs in necrosis-inducing inflammatory processes.

MT-1 cells express Fas protein on the cell surface. When these cells were stimulated with anti-Fas Ab, which activates the Fas-mediated apoptotic pathway, approximately 30% of the cells induced apoptosis within 4 hr (Fig. 1). In contrast, when they were allowed to stand for 15 hr with anti-Fas-Ab, approximately 40% of the cells turned necrotic as proven judged by PI staining (Fig. 1). The mAbs were produced by periodically immunizing Balb/c mice by injection of $10^8$ 15 hr-Fas-sensitized cells per mouse into their peritoneal cavities, as reported previously (10). Six such immunizations enabled the mice to produce mAbs that recognized the immunogenicity of MT-1 cells. Using immunoblotting analysis, we screened the supernatants of >1200 clones of hybridoma cells and identified mAbs that specifically recognized MT-1 cells in a mixture of live, apoptotic and necrotic cells. Ten of them were reacted with the cell mixture (data not shown). We next checked whether each mAb recognized apoptotic or necrotic cells. Apoptotic and necrotic cells were prepared separately and the reactivity of these 10 mAbs to these cells was examined by flow cytometry. No mAb recognized apoptotic MT-1 cells, and only three mAbs recognized necrotic MT-1 cells (Fig. 2). The other mAbs recognized some cytoplasmic proteins of live MT-1 (data not shown). Of the three mAbs that bound necrotic cells, #74 was IgG1 while #121 and #170-1 were IgM.

We next tested the kind of molecules recognized by these three mAbs, using immunoblotting. mAbs #74 and #121 reacted with an 80 kDa protein on the Western blot of a MT 1 cell homogenate (Fig. 3b). This protein appeared in both live and dead cells, suggesting that the protein had been expressed on the cell surface or had become accessible to mAb as a result of necrotic events. mAb #170-1 also bound an 80 kDa protein and two other moieties of 53 kDa and 41 kDa on the blot (Fig. 3b). This mAb presumably recognizes a single epitope in a protein. If so, the latter two moieties must be degradation products of the 80 kDa protein. Although it is important to identify the antigenic protein(s) of 80 kDa, N-terminal analysis did not correspond to any known protein (data not shown).

Finally, we confirmed that these mAbs specifically interact with necrotic MT-1 cells. Figure 3a shows an example analysis of #121 mAb, indicating that #121 recognizes necrotic cells but not live MT-1 cells, a finding which is in agreement with the results of the fluorogram (see Fig. 2). Although this is not shown in Figure 3, apoptotic cells failed to interact with #121, consistent with the results shown in Figure 2. Similar results were obtained with the other two mAbs (data not shown). Hence we have successfully established one IgG1 and two IgM mAbs that exclusively gain access to necrotic cell antigen(s).

Ab and complement form an immune complex with affected cells and amplify local inflammation. Macrophages and dendritic cells phagocytose antigens of altered cells and facilitate antigen-specific lymphocyte activation. The present study may be interpreted as showing that necrotic

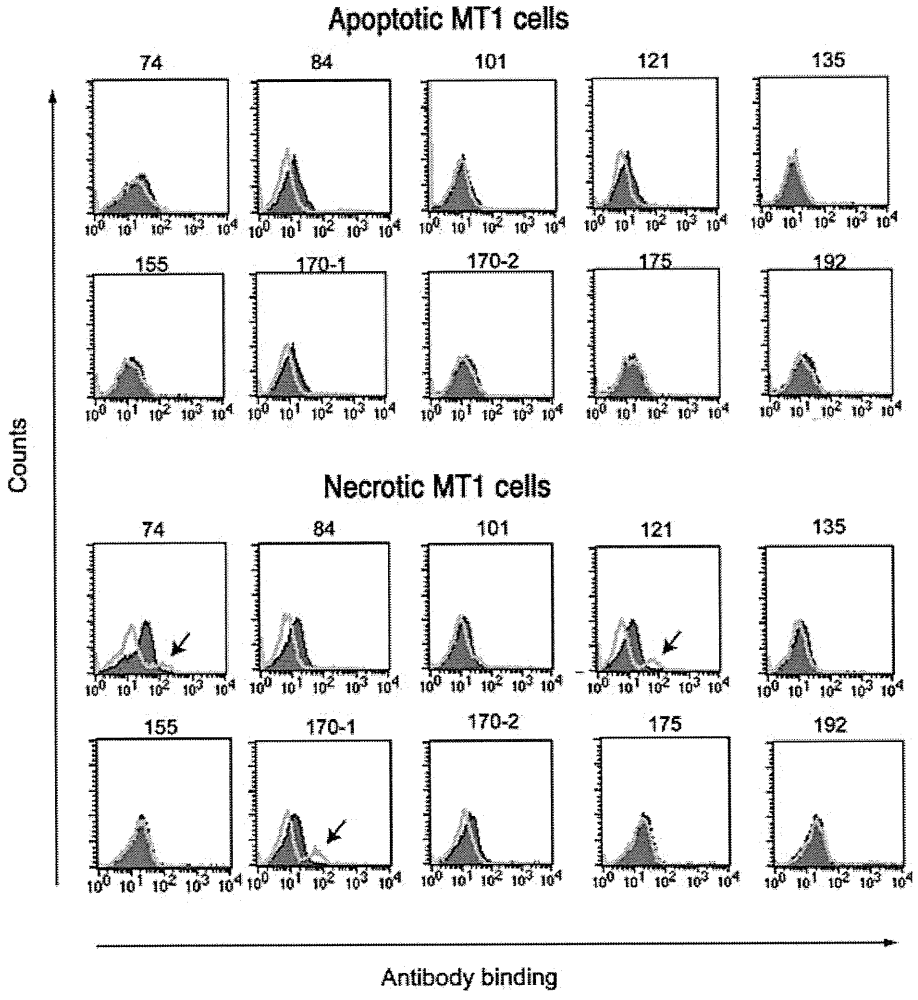


**Fig. 2. Screening for mAbs that specifically recognize necrotic MT1 cells.** MT1 cells treated with 50 ng/mL anti-Fas antibody for 4 hr (upper panels, labeled Apoptotic) and 15 hr (lower panels, labeled Necrotic) were incubated with the supernatants of hybridomas. Alexa 488-labeled secondary antibody was used as a detector for flow cytometry. The results of the final screening are shown as fluorometry. Gray histograms, control non-immune first Ab; open histograms, specific first mAbs. As indicated by arrows, three mAbs #74, #121 and #170-1 formed two peaks, reflecting interaction with necrotic cells.

cells participate in the activation of cellular immunity by expressing specific antigens on their cell surface, and that this reflects a specific stage of inflammation. The results of our screening of mAbs against MT-1 suggest that such necrosis-specific antigens become accessible in the cells and can be detected by our mAbs. Not only IgM, but also IgG1, mAbs recognize the surface-accessible antigens expressed on the dead MT-1 cells.

How the molecules in necrotic cells become accessible to mAbs is a crucial point. We propose that: (i) the tight lipid bilayer in live cells loosens in necrotic cells, allowing mAbs to interact with inner membrane molecules; or (ii) membrane flip-flop occurring in necrotic cells increases the accessibility of mAbs to inner membrane proteins (11, 12). Although we do not have precise evidence for how intracellular proteins appear on the surface of necrotic cells, the resultant cellular proteins are of limited types and have membrane-associated properties. Studies are in progress to determine whether the 80 kDa protein we found has DAMP properties *per se*.

We initially tried to establish a homogenous system consisting of mouse tumor cells, mouse mAb and syngeneic mouse macrophages/dendritic cells. However, we could not obtain mAb against autologous cells despite a number of trials. Reis e Sousa *et al.* have shown that a xeno-system works well in the evaluation of innate cell activation by virus-infected cells (13). In their study, double-stranded RNA replicated in the virus-infected human cells was a

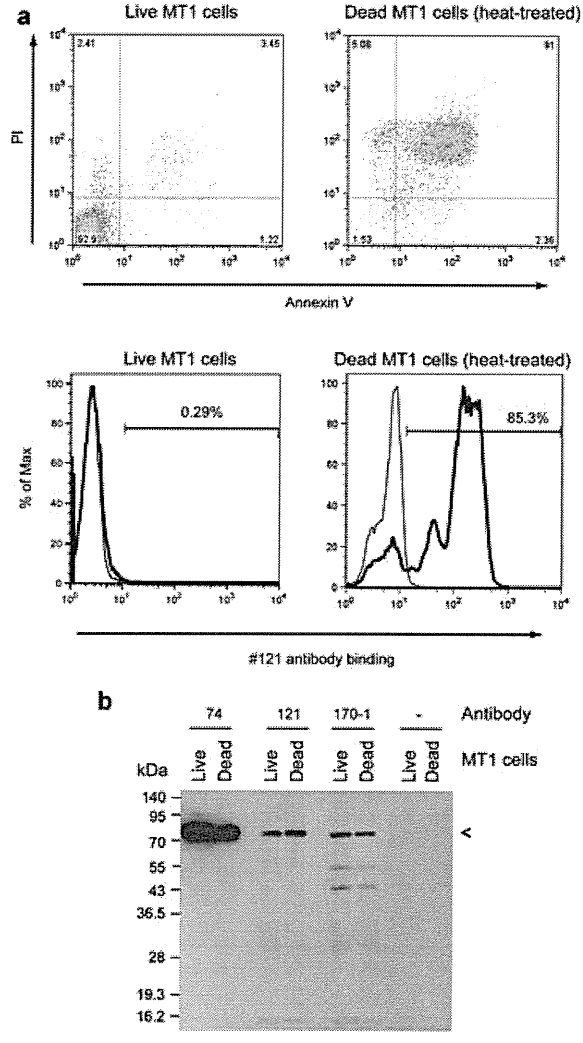


**Fig. 3. Detection of cellular antigens by mAbs.** (a) Flow cytometric analysis for detection of #121 antigen in heat treatment-induced necrotic MT1 cells. MT1 cells were treated with 52°C for 20 min to induce artificial necrotic cell death. The necrotic MT1 cells were stained with #121 antibody and Alexa 488-labeled secondary antibody. Surface-accessible antigens were detected by flow cytometry. Similar results were obtained with the other two mAbs. (b) Immunoblotting analysis. Lysates of live or necrotic MT1 cells were subjected to SDS-PAGE followed by immunoblotting with mAbs as indicated. The arrow head indicates the 80 kDa antigenic protein.

pattern molecule for mouse TLR3 *in vitro* and *in vivo* (13). According to the pattern-sensing system, the properties of pattern molecules rather than the origin of cell debris are crucial for the induction of phagocyte-mediated innate immune response (14). Thus, we decided to use an immune complex consisting of human MT-1 cells and mouse mAbs to analyze the properties of immune complex-derived inflammation in mice. This is the first report of establishment of a system for evaluation of Ab effects on DAMP function under necrotic conditions.

Other investigators have reported that inflammation acts on the immune system as well as on cancer and infected cells (15, 16). Pattern molecules are representative ligands for PRRs, but once necrotic cells have emerged inflammation is self-amplified, resulting in the formation of an exacerbation loop. It is well known that Ab and complement accelerate such self-amplification loops (17). Many factors are associated with the loop formation secondary to immune complex formation, however most of them remain undetermined. Our system using anti-necrosis cell-specific mAbs may contribute to the identification of host factors that participate in DAMP-mediated augmentation of inflammatory responses.

DAMP may modulate cell growth and immune response in cancer and infectious nests (1, 2, 14). By injection of mAb and necrotic MT-1 into mice, we will be able to test the *in vivo* events induced by tumor-derived immune modulation. The establishment and availability of these mAbs will make many trials for the analysis of sequential events in the areas of immune response in cancer and other microbial infections feasible in future.

## ACKNOWLEDGMENTS

We are grateful to Dr. Vijaya Kumar for his English editing. Thanks are also due to Drs. J. Kasamatsu, K. Hazeki, N. Inoue, T. Akazawa and H. Oshiumi for valuable discussions. This work was in part supported by Grants-in-Aid from the Ministry of Education, Science, and Culture, the Ministry of Health, Labor, and Welfare of Japan, and by the Waxman Foundation and the Akiyama Life Science Foundation.

## REFERENCES

1. Kono H., Rock K.L. (2008) How dying cells alert the immune system to danger. *Nat Rev Immunol* **8:** 279–89.
2. Seong S.Y., Matzinger P. (2004) Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol* **4:** 469–78.
3. Shi Y., Evans J.E., Rock K.L. (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425:** 516–21.
4. Yanai H., Ban T., Wang Z., Choi M.K., Kawamura T., Negishi H., Nakasato M., Lu Y., Hangai S., Koshiba R., Savitsky D., Ronfani L., Akira S., Bianchi M.E., Honda K., Tamura T., Kodama T., Taniguchi T. (2009) HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature* **462:** 99–103.
5. Srikrishna G., Freeze H.H. (2009) Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. *Neoplasia* **11:** 615–28.
6. Sato S., St-Pierre C., Bhaumik P., Nieminen J. (2009) Galectins in innate immunity: dual functions of host soluble

beta-galactoside-binding lectins as damage-associated molecular patterns (DAMPs) and as receptors for pathogen-associated molecular patterns (PAMPs). *Immunol Rev* **230:** 172–87.
7. Du F., Nakamura Y., Tan T.L., Lee P., Lee R., Yu B., Jamora C. (2010) Expression of snail in epidermal keratinocytes promotes cutaneous inflammation and hyperplasia conducive to tumor formation. *Cancer Res* **70:** 10080–9.
8. Rees R.F., Gewurz H., Siegel J.N., Coon J., Potempa L.A. (1988) Expression of a C-reactive protein neoantigen (neo-CRP) in inflamed rabbit liver and muscle. *Clin Immunol Immunopathol* **48:** 95–107.
9. Gabrilovich D.I., Nagaraj S. (2009) Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* **9:** 162–74.
10. Hara T., Matsumoto M., Tsuji S., Nagasawa S., Masaoka T., Kodama K., Horai T.,Sukuma T., Seya T. (1996) Enhanced opsonization of drug-induced apoptotic cells of a human adenocarcinoma cell line by homologous complement activation. *Immunobiol* **196:** 491–503.
11. Nusbaum P., Lainé C., Seveau S., Lesavre P., Halbwachs-Mecarelli L. (2004) Early membrane events in polymorphonuclear cell (PMN) apoptosis: membrane blebbing and vesicle release, CD43 and CD16 down-regulation and phosphatidylserine externalization. *Biochem Soc Trans* **32**(Pt 3): 477–9.
12. Hoffman T., Hirata F., Bougnoux P., Fraser B.A., Goldfarb R.H., Herberman R.B., Axelrod J. (1981) Phospholipid methylation and phospholipase A2 activation in cytotoxicity by human natural killer cells. *Proc Natl Acad Sci U S A* **78:** 3839–43.
13. Schulz O., Diebold S.S., Chen M., Näslund T.I., Nolte M.A., Alexopoulou L., Azuma Y.T., Flavell R.A., Liljeström P., Reis e Sousa C. (2005) Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* **433:** 887–92.
14. Chen C.J., Kono H., Golenbock D., Reed G., Akira S., Rock K.L. (2007) Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* **13:** 851–6.
15. Killeen S.D., Wang J.H., Andrews E.J., Redmond H.P. (2006) Exploitation of the Toll-like receptor system in cancer: a doubled-edged sword? *Br J Cancer* **95:** 247–52.
16. Conroy H., Marshall N.A., Mills K.H. (2008) TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours. *Oncogene* **27:** 168–80.
17. Chen Y., Khanna S., Goodyear C.S., Park Y.B., Raz E., Thiel S., Grönwall C., Vas J., Boyle D.L., Corr M., Kono D.H., Silverman G.J. (2009) Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol* **183:** 1346–59.

# 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 Yamazaki[1*☯], Kohei Okada[1☯], Akira Maruyama[1], Misako Matsumoto[1], Hideo Yagita[2], Tsukasa Seya[1*]**

**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 vitro*. 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 depletion 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 Santé (CRP-Santé), Luxembourg

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



**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 vitro* [25] and that local injection of Pam2 lipopetides 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 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 TLR2 because T reg cells were not increased in TLR2KO mice injected with Pam2CSK4 (Fig. 3B).

To investigate whether the increase of $Foxp3^+$ T reg is 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 naïve 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 naïve mice or Pam2

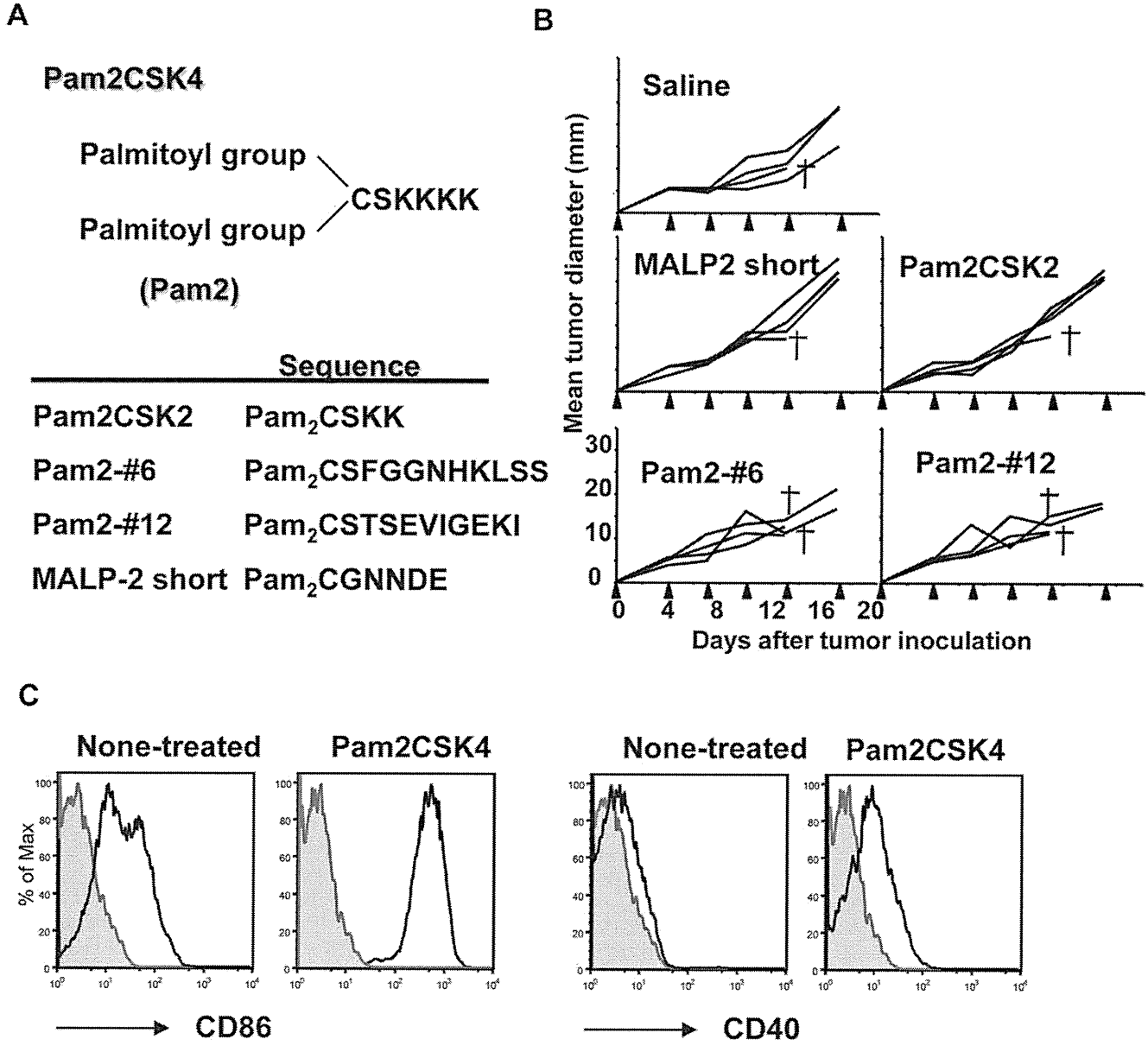


**Figure 1. Pam2 lipopeptides do not induce effective anti-tumor immunity.** (A) Structures of the Pam2 lipopeptides are shown. (B) Mice were injected with B16D8 melanoma cells ($2\times10^5$) on back. The mice were injected s.c. into their footpad with the indicated Pam2 lipopeptides (10 nmol) or saline 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

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-CD25mAb (PC61) 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.

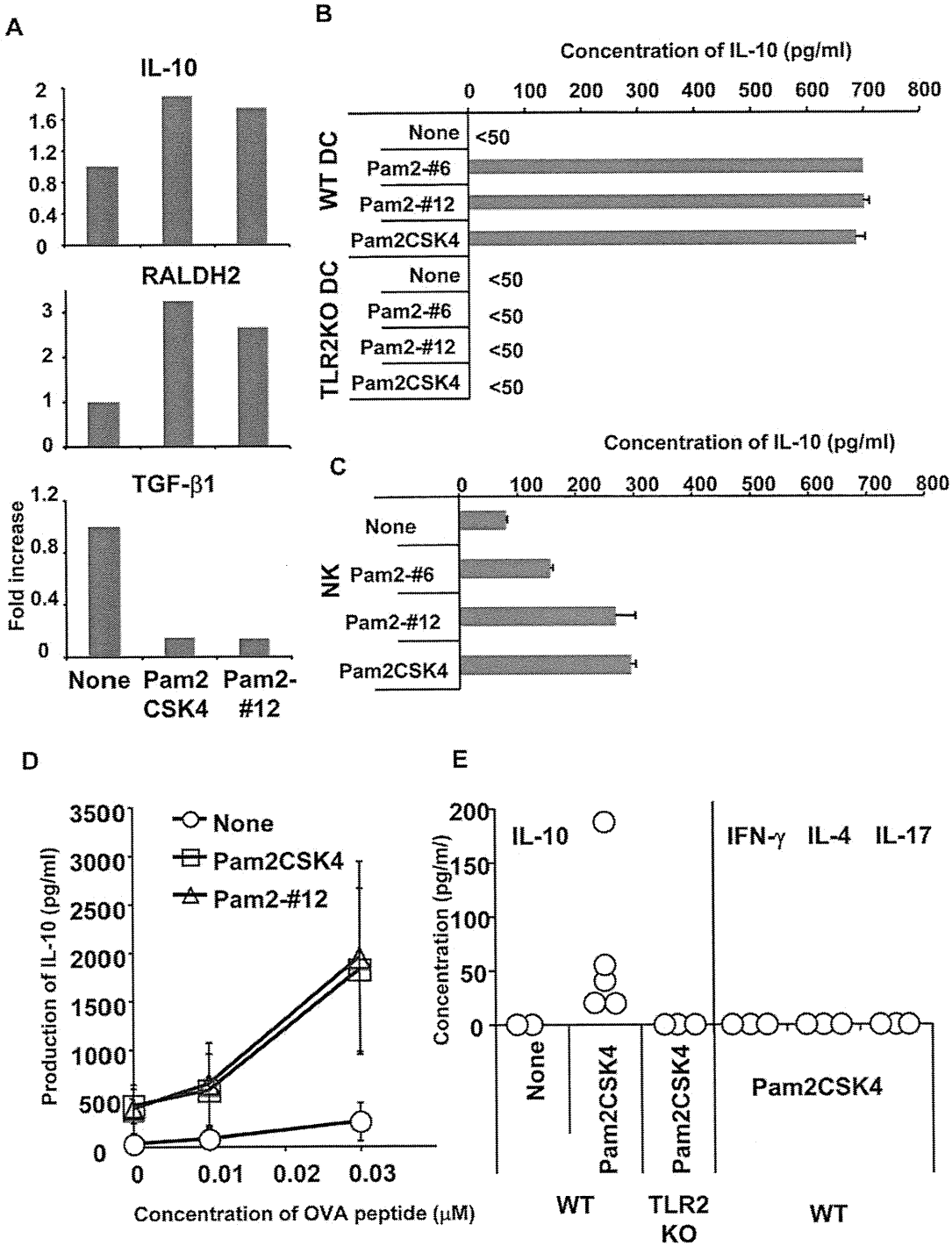


**Figure 2. Pam2 lipopeptides induce IL-10 and retinal dehydrogenase.** (A) Spleen DCs from B6 mice were cultured with or without 100 nM of Pam2CSK4 or Pam2-#12. After four hours, total RNA was prepared and real-time PCR was performed. Expression of each sample was normalized to GAPDH mRNA expression and fold increases of each sample were calculated to the expression levels at 0 hours. One of two experiments is shown. (B) BM-DCs ($1\times10^5$) 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 ($2\times10^5$) 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 ($5\times10^4$) were cultured with spleen DCs ($5\times10^4$) 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

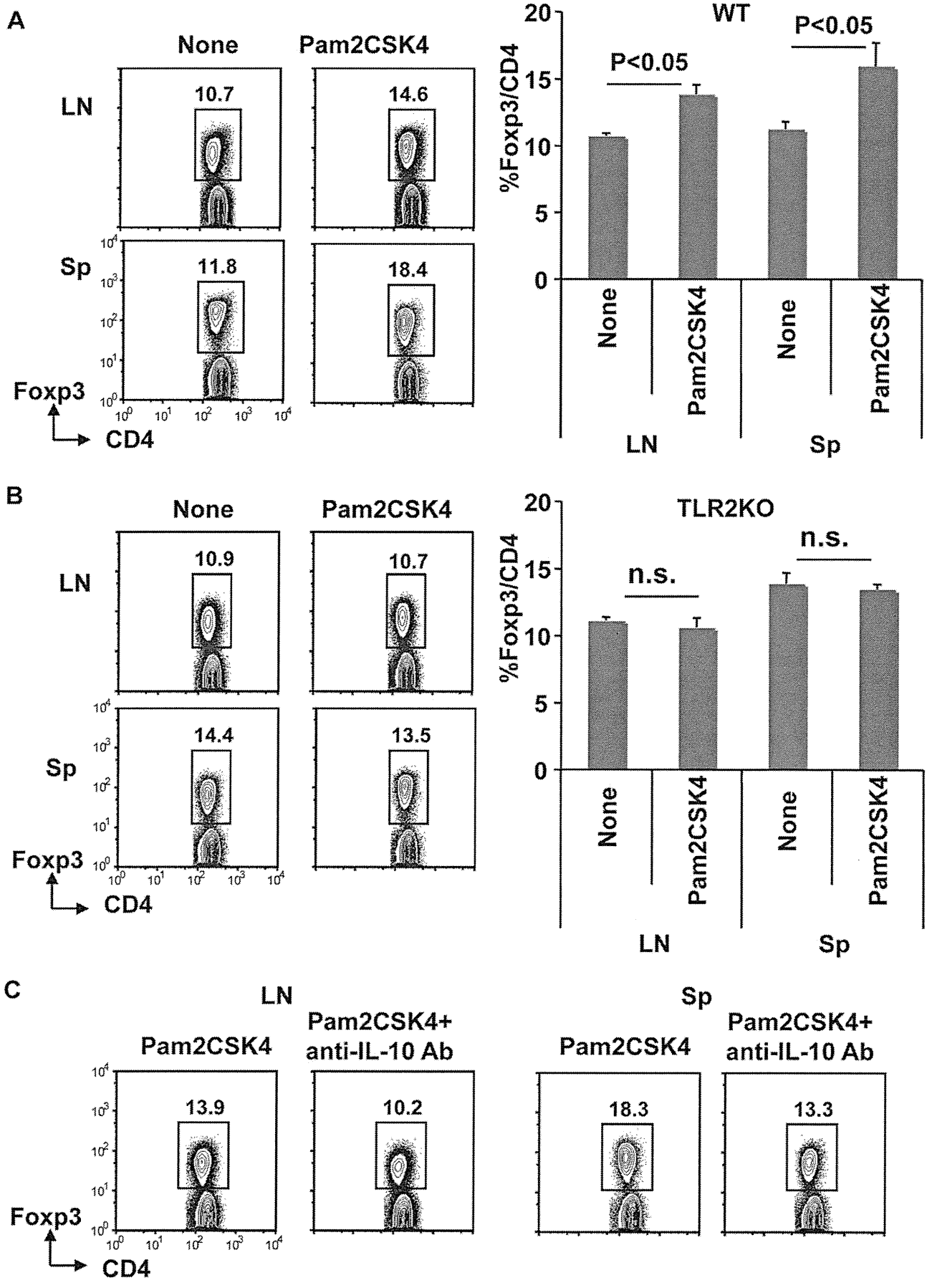
A
None
Pam2CSK4
LN
Sp
10.7
14.6
11.8
18.4
Foxp3
CD4
WT
%Foxp3/CD4
P<0.05
P<0.05
None
Pam2CSK4
None
Pam2CSK4
LN
Sp
B
None
Pam2CSK4
LN
Sp
10.9
10.7
14.4
13.5
Foxp3
CD4
TLR2KO
%Foxp3/CD4
n.s.
n.s.
None
Pam2CSK4
None
Pam2CSK4
LN
Sp
C
LN
Sp
Pam2CSK4
Pam2CSK4+
anti-IL-10 Ab
Pam2CSK4
Pam2CSK4+
anti-IL-10 Ab
13.9
10.2
18.3
13.3
Foxp3
CD4

**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. The image summarizes the results from two separate experiments. N.s. stands for not significant according to the student's-t test. (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$^+$ cytotocxic 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 to inducing tolerance in the case of autoimmunity, 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 [23,38]. 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 vitro* (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 maintained the suppressive function of T reg cells and promoted their survival [37]. This discrepancy might be caused by the use of CD25$^+$ CD4$^+$ T cells contaminated with Foxp3$^-$CD25$^+$CD4$^+$ T cells, and Foxp3-GFP reporter mice [37]. Contamination of Foxp3$^-$CD25$^+$CD4$^+$ T cells could affect the results, especially when TLR2 ligands were continuously present in the culture, since TLR2 is also expressed on activated effector T cells. In this report, we stimulated T reg cells with Pam2 lipopeptides *in vivo* and purified T reg cells as CD25$^+$ CD4$^+$ T cells because Foxp3-GFP reporter mice were not available. However, CD25$^+$ CD4$^+$ T reg cells purified from Pam2CSK4 treated mice were as suppressive as T reg cells from naïve mice (Fig. 4B). This indicated that T reg cells stimulated by TLR2 did not reverse the suppressive function *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.

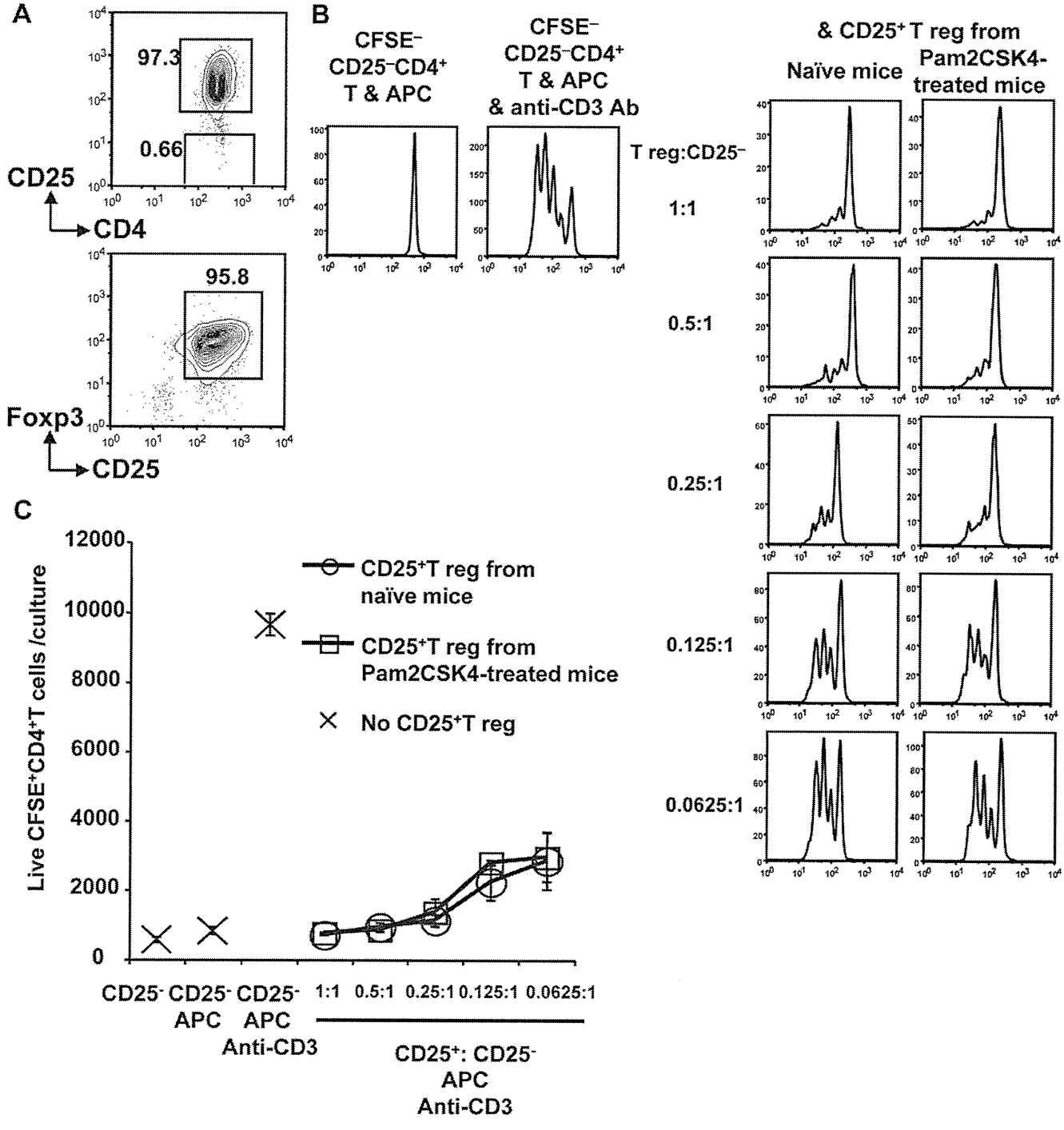


**Figure 4. T reg cells from Pam2CSK4-treated mice maintain suppressive activity.** (A)$CD25^+CD4^+$ T cells purified by flow cytometry were further fixed and stained with Foxp3. FACS plots were gated on $CD4^+$ T cells. One of three similar experiments is shown.(B) B6 mice were i.p. injected with Pam2CSK4 (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 ($5\times10^4$) were stimulated with irradiated spleen antigen presenting cells ($1\times10^5$) 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

## 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

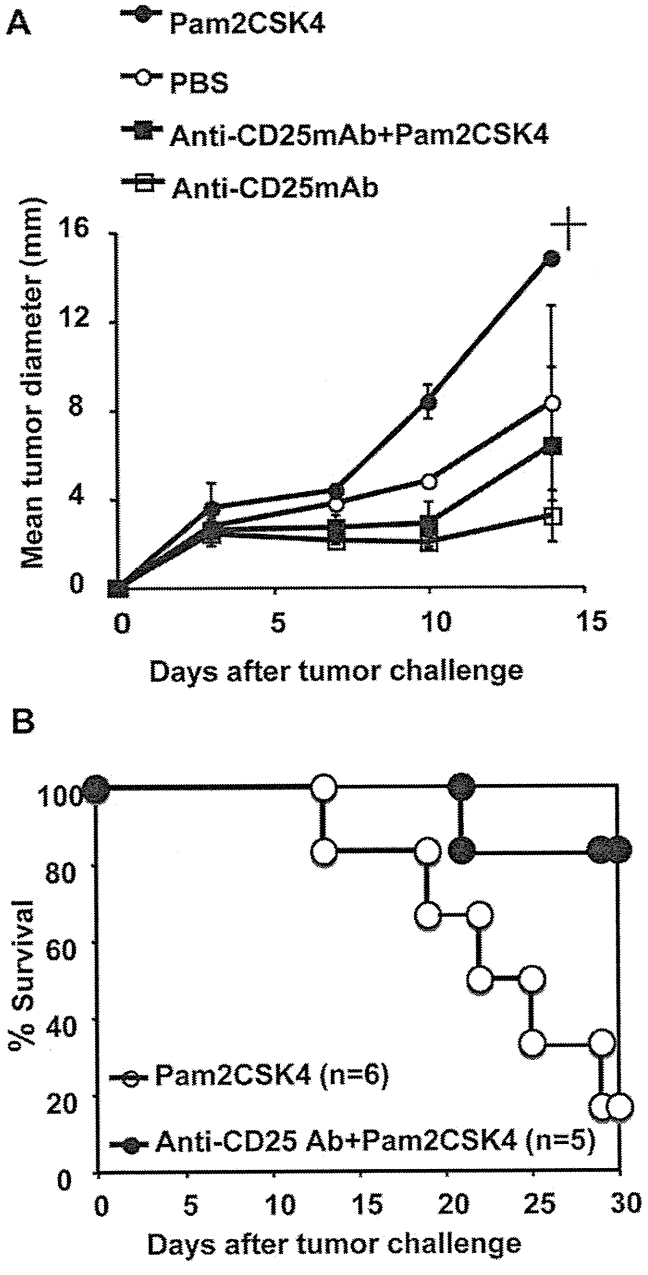


**Figure 5. NK-sensitive tumors grow slowly when Pam2CSK4 is injected into T reg-depleted mice.** (A) B6 mice were i.p. injected with 500 μg of anti-CD25mAb (PC61) on day -3. The mice were injected with B16D8 melanoma cells ($2\times10^5$) into their back on day 0. Pam2CSK4 (10 nmol) or PBS was injected 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

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 $2-3\times10^5$ 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

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 15–20 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. After 16 hours, splenic NK cells were analyzed by flow cytometry. Plots were gated on $NK1.1^+$ cells.
(TIF)

**Figure S2 The frequency of T reg cells returns to normal at day 7 after systemic injection of Pam2 lipopeptides.** WT mice were i.p. injected with Pam2CSK4 (10 nmol). After seven 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 two experiments is shown for the FACS plots.
(TIF)

## Acknowledgments

We thank Ms. Ryoko Sawahata for technical assistance at the beginning of the project, Dr. Hussein Aly for reading the manuscript, Dr. Kazuya Iwabuchi and Dr. Shizuo Akira for the mice, Dr. Yukari Fujimoto and Dr. Koichi Fukase for the peptides, Dr. Jun Shimizu for providing the hybridoma cells and Dr. Ralph Steinman for providing the antibody.

## Author Contributions

Conceived and designed the experiments: SY MM TS. Performed the experiments: SY KO AM. Analyzed the data: SY KO. Contributed reagents/materials/analysis tools: HY. Wrote the paper: SY TS.

## 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, Yamazaki S, Nakamura K, Nishioka T, Hirota K, et al. (2005) 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: 885–891.
4. Li X, Kostareli E, Suffner J, Garbi N, Hammerling GJ (2010) Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. Eur J Immunol 40: 3325–3335.
5. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, et al. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10: 942–949.
6. Ghiringhelli F, Puig 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, Yamazaki 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. Onizuka S, 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: 3128–3133.
9. Teng MW, Swann JB, von Scheidt B, Sharkey J, Zerafa N, et al. (2010) Multiple antitumor mechanisms downstream of prophylactic regulatory T-cell depletion. Cancer Res 70: 2665–2674.
10. Sutmuller RP, van Duivenvoorde 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 GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. J Clin Invest 116: 1935–1945.
12. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP (2009) Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J Exp Med 206: 1717–1725.
13. Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, et al. (2010) Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res 70: 7788–7799.
14. Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. Nature 449: 419–426.
15. 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.
16. Dubensky TW, Jr., Reed SG (2010) Adjuvants for cancer vaccines. Semin Immunol 22: 155–161.
17. Seya T, Matsumoto M (2009) The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 58: 1175–1184.
18. Yamazaki S, Iyoda 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. Yamazaki S, Patel M, Harper A, Bonito A, Fukuyama 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-Guerin: involvement of toll-like receptors. Infect Immun 68: 6883–6890.
21. Azuma I, Seya T (2001) Development of immunoadjuvants for immunotherapy of cancer. Int Immunopharmacol 1: 1249–1259.
22. Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, et al. (2007) Antitumor NK activation induced 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, Tsuji S, Akazawa 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-Guerin peptidoglycan. Infect Immun 71: 4238–4249.
24. Murata M (2008) Activation of Toll-like receptor 2 by a novel preparation of cell wall skeleton from Mycobacterium bovis BCG Tokyo (SMP-105) sufficiently enhances immune responses against tumors. Cancer Sci 99: 1435–1440.
25. Azuma M, Sawahata R, Akao Y, Ebihara T, Yamazaki S, et al. (2010) The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation. PLoS One 5: e12550.
26. Akazawa 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: 1596–1603.
27. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, et al. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg 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 mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 204: 1757–1764.
29. Manicassamy S, Ravindran R, 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, Chaturvedi 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. Yamazaki S, Bonito AJ, Spisek R, Dhodapkar 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: 28–50.
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: 4458–4466.
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: 1075–1085.
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 cross-talk in lymph nodes at the steady state by inhibiting CD4+ self-reactive T cells. J Immunol 180: 4679–4686.
42. Mehrotra PT, Donnelly RP, Wong S, Kanegane H, Geremew 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, Smyth 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, Erten G, Kucuksezer UC, Kocacik D, Karagiannidis C, et al. (2008) Regulatory NK cells suppress antigen-specific T cell responses. J Immunol 180: 850–857.
46. Lee SH, Kim KS, Fodil-Cornu N, Vidal SM, Biron CA (2009) Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. J Exp Med 206: 2235–2251.
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, Stoelcker B, Kohler M, et al. (2010) Interleukin-10 promotes NK cell killing of autologous macrophages by stimulating expression of NKG2D ligands. Scand J Immunol 72: 319–331.
49. Alter G, Kavanagh D, Rihn S, Luteijn 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, Luhrmann A, Grote K, Tschernig T, et al. (2003) Intratracheal macrophage-activating lipopeptide-2 reduces metastasis in the rat lung. Am J Respir Cell Mol Biol 28: 316–321.
51. Schmidt J, Welsch T, Jager D, Muhlradt PF, Buchler MW, et al. (2007) Intratumoural injection of the toll-like receptor-2/6 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 LW, et al. (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 116: 485–494.
53. Liu H, Komai-Koma M, Xu D, Liew FY (2006) Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ 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 alloantigen. Transplantation 79: 568–576.
55. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, et al. (2008) CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. J Immunol 181: 6923–6933.