

DX5⁺ NKG2D⁺ NK cells were present in the spleens of CB6F₁ mice even before FV infection, consistent with the high baseline NK activities detectable in CB6F₁ mice (Fig. 1A). The proportion of NKG2D⁺ cells among CD8⁺ T cells remained low until PID 8, but the absolute numbers of NKG2D⁺ CD8⁺ cells abruptly increased by PID 12.

As to the expression of NKG2D ligands on FV-infected cells *in vivo*, flow cytometric analyses revealed increased expression of RAE-1 and MULT1, but not H60, on the surfaces of gp70⁺ and TER-119⁺ erythroid progenitor cells in the spleens of FV-infected CB6F₁ mice (Fig. 3A). As shown in Fig. 3A, gp70⁺ cells were only slightly increased in the spleen until PID 8. Nevertheless, >1/5 of the gp70⁺ cells expressed RAE-1 above the level of the demarcation line, while a large majority of gp70⁻ cells were also negative for RAE-1. By PID 12, gp70⁺ populations increased drastically, and a significant proportion of them expressed RAE-1 above the level of the demarcation line. At the same time point, three distinctive populations of gp70⁺ cells with undetectable levels of TER-119 expression (TER-119⁻ gp70⁺ cells), low levels of TER-119 and high gp70 expression (TER-119^{Lo} gp70^{Hi} cells, purple dots, Fig. 3A), and TER-119^{Hi} gp70⁺ erythroblasts (red dots, Fig. 3A) appeared in the spleen. Based on a previous report (50), these three populations of gp70⁺ cells most likely represent distinctive stages of erythroid cell differentiation from FV-infected progenitor cells, including primitive erythroid progenitor cells and proerythroblasts (TER-119⁻), proerythroblasts and early basophilic erythroblasts (TER-119^{Lo}), and maturing benzidine-positive erythroblasts (TER-119^{Hi}). Importantly, the TER-119^{Lo} population that showed higher levels of gp70 expression than the two other populations also showed higher levels of RAE-1 and MULT1 expression on their surfaces, but their levels of H60 expression were below the detection limit (Fig. 3A).

When mean fluorescence intensities were compared between different populations of spleen cells, the average levels of RAE-1 expression on the surfaces of gp70⁺ cells were significantly higher than those on gp70⁻ cells in CB6F₁ mice at all time points tested (Fig. 4). Further, TER-119^{Lo} proerythroblasts and early basophilic erythroblasts expressed significantly higher levels of RAE-1 on their surfaces than TER-119⁻ cells at PID 12, indicating that FV-infected erythroid cells can become susceptible to NK killing due to RAE-1 upregulation.

Decreased MHC class I and class Ib antigen expression on terminally differentiating erythroid cells. The target-killing activities of NK cells are regulated not only by the above positive signals but also by negative signals generated by receptors binding to MHC molecules, and the MHC class Ib Qa-1^b molecule is a strong inhibitor of NK activities in mice (49). Thus, FV-infected erythroid cells might also become susceptible to NK killing due to their possibly reduced expression of class I and/or class Ib molecules. As shown in Fig. 3B, the TER-119⁻ population of spleen cells retained high levels of MHC class I K^b and lower levels of D^b molecules, as well as class Ib Qa-1^b, on their surfaces until PID 12; however, decreased expression of Qa-1^b, as well as K^b and D^b, was discernible in a small population of TER-119⁺ erythroid cells at PID 4 and 8 (data not shown). At PID 12, TER-119^{Hi} terminally differentiating erythroid cells (Fig. 3B, pink dots) showed markedly diminished expression of the class I molecules, and

Qa-1^b expression on the majority of TER-119^{Hi} cells became barely detectable. It is notable, however, that the TER-119^{Lo} cells that showed increased expression of RAE-1 (Fig. 3A) retained unchanged levels of class I and Qa-1^b expression, and the lack of Qa-1^b expression was observed only with the TER-119^{Hi} population. As levels of RAE-1 and MULT1 expression, as well as those of viral gp70, on the surfaces of TER-119^{Hi} cells were also reduced in comparison with those on TER-119^{Lo} cells at PID 12 (Fig. 3A), these observations may reflect the global downregulation of gene expression in terminally differentiating erythroid cells (33, 40). Thus, gp70^{Hi} TER-119^{Lo} erythroblasts that are massively increased in the spleen by PID 12 express higher levels of RAE-1 and MULT1 but retain the expression of Qa-1^b.

***In vivo* role of NKG2D–RAE-1 interactions in controlling early expansion of FV-infected cells and the development of virus-induced pathologies.** Although the above experiments have shown significant expansion of NKG2D⁺ NK cells and increased expression of RAE-1 proteins on infected erythroid cells upon FV inoculation, the sufficiency of the levels of RAE-1 expression induced in TER-119^{Lo} erythroid cells to evoke NK-mediated killing in the presence of Qa-1^b can be questioned. In this regard, the Ab-mediated depletion of NK cells from mice immunized with a single-epitope peptide vaccine totally abrogated the protective efficacy of Th cell priming, and NK-depleted mice died as rapidly as unimmunized control mice after FV infection in the previous experiments (16). However, the possible physiological role of NK cells in regulating the early proliferation of FV-infected erythroid cells in unimmunized mice of a susceptible strain has not been analyzed. To address this, we administered anti-asialoGM1 Ab to unimmunized CB6F₁ mice and reduced the percentages of DX5⁺ NK-1.1⁺ NK cells in the spleen to <1.2 through PID 4 to 12. In the above NK-depleted mice, the number of FV infectious centers in the spleen drastically increased at as early as PID 6 (Fig. 5A), indicating that NK cells are actually restricting early expansion of FV-infected cells in the spleen. The percentages of gp70⁺ cells in the spleen at 6 days after FV infection also increased significantly in the NK-depleted mice and became 3 times higher than those in the control mice infected without NK depletion (Fig. 5B). As CB6F₁ mice are highly susceptible to FV-induced disease development, the percentages of gp70⁺ cells in the spleen increased toward PID 12 in both groups; however, the proportions of gp70⁺ cells further increased in the absence of asialoGM1⁺ cells in comparison with those in the control animals given unimmunized sera. Importantly, NK cell depletion also affected the survival of FV-infected mice: when inoculated with a slightly reduced dose of 50 SFFU, highly susceptible CB6F₁ mice still developed the fatal disease and most died by 60 days after FV infection, while NK-depleted CB6F₁ mice died significantly more rapidly, with an average survival period that was 9 days shorter than that of the control mice (Fig. 5C). These results indicate that NK cells are not only involved in confining the early expansion of FV-infected erythroid cells, but are contributing to natural resistance to FV-induced disease development in unimmunized animals.

Although the administration of anti-asialoGM1 Ab did not affect CD4⁺ and CD8⁺ T-cell numbers and their functionalities in the previous experiments (16), it is possible that FV-reactive T cells, along with NK cells, might have been affected

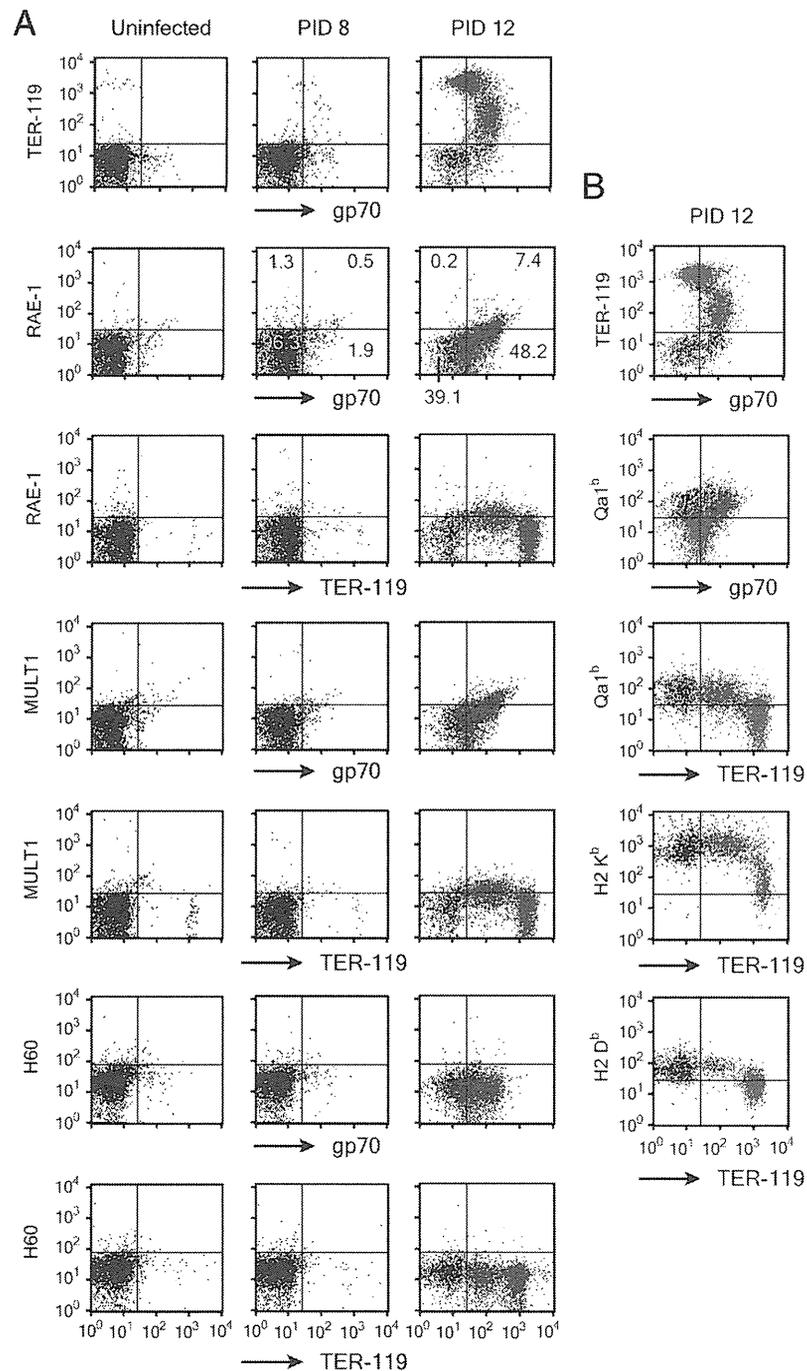


FIG. 3. Representative dot plots showing the expression of NK receptor ligand proteins (A) and MHC class I and class Ib molecules (B) on the surfaces of spleen cells in FV-infected CB6F₁ mice. CB6F₁ mice were inoculated with 150 SFFU FV, and at least 4 mice were killed at PID 4, 8, and 12 to remove the spleen. Multicolor flow cytometric analyses were performed with each indicated Ab. Uninfected mice were similarly analyzed as controls. Mature erythrocytes and dead cells were excluded by setting a polygonal gate in the dot plots showing intensities of forward scatter and the fluorescence for 7-aminoactinomycin D. Demarcation lines were set based on the fluorescence levels observed by incubating the cells with isotype control Ab. Data on PID 4 are omitted from this figure, as they are essentially similar to those on PID 8, except that gp70⁺ populations are much smaller. In the middle and right panels of the second row of dot plots in panel A, the number in each quadrant represents the percentage of cells that expressed the indicated markers above or below the level of the corresponding demarcation line. Patterns observed with all 4 mice at each time point were consistent with those shown here. In panel A, purple dots, TER-119^{lo} gp70^{hi} cells; red dots, TER-119^{hi} gp70⁺ erythroblasts. In panel B, red dots, TER-119^{lo} gp70^{hi} cells; pink dots, TER-119^{hi} gp70⁺ erythroblasts.

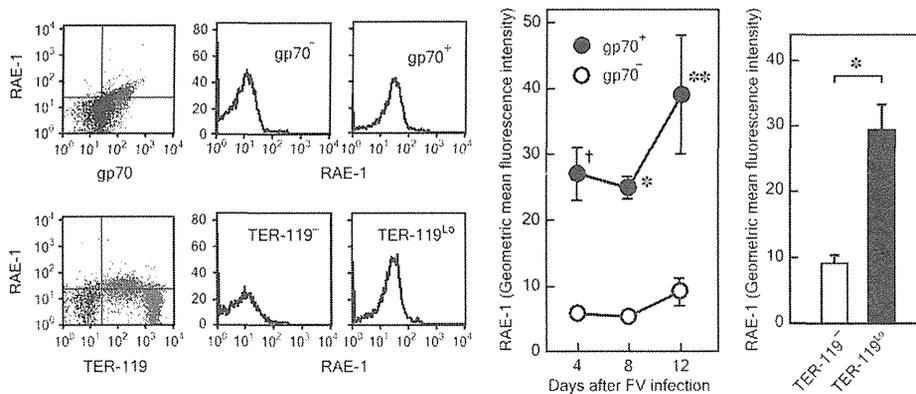


FIG. 4. Different levels of RAE-1 expression between viral gp70-positive and -negative spleen cells in mice infected with FV. CB6F₁ mice were inoculated with 150 SFFU of LDV-free FV, and multicolor flow cytometric analyses were performed on nucleated cells as described for Fig. 3. Representative dot plots and histograms on the left show different levels of RAE-1 expression on pairs of gp70⁺ and gp70⁻ or TER-119⁻ and TER-119^{Lo} populations at PID 12. TER-119⁻ and TER-119^{Lo} populations are those shown with black and purple dots, respectively, in Fig. 3A. Changes in averages of geometric mean fluorescence intensity (GMFI) for RAE-1 among gp70⁻ and gp70⁺ cells after FV infection are shown in the middle panel. GMFI were calculated by using the region statistics function of the CellQuest software. Each datum shown here is the mean ± SEM, calculated with GMFI data obtained from 4 mice. Differences between gp70⁺ and gp70⁻ populations were examined by two-way ANOVA with Bonferroni *post hoc* tests: *, $P < 0.05$; †, $P < 0.01$; **, $P < 0.001$. The right panel shows averages of GMFI for RAE-1 compared between the TER-119⁻ and TER-119^{Lo} populations at PID 12. Each bar shows mean ± SEM calculated with GMFI data obtained from 4 or 5 mice. *, $P < 0.02$, by paired *t* test.

by the Ab injections in the above depletion experiments, as activated T cells are known to express asialoGM1 in some viral infections (41). To further demonstrate the direct involvement of the RAE-1 ligand and NKG2D receptor in confining the early expansion of FV-infected cells, blocking Abs were administered to infected CB6F₁ mice. The numbers of FV-producing cells in the spleen significantly increased in the animals that were given either NKG2D- or RAE-1-blocking Ab in comparison with those in the control mice (Fig. 5A and D). Further, in the presence of the RAE-1-blocking Ab, FV-infected CB6F₁ mice showed significantly larger spleen sizes than control mice (Fig. 5E), confirming that FV-infected erythroid progenitor cells are recognized and their expansion is regulated through NKG2D–RAE-1 interactions *in vivo*. As the effects of the NKG2D or RAE-1 blockade were observed at as early as PID 6, prior to the expansion of NKG2D⁺ CD8⁺ T cells (Fig. 1B), it is most likely that mainly NKG2D⁺ NK cells were responsible for the above early confinement of FV-induced erythroid cell proliferation.

Increased expression of RAE-1 depends on retroviral replication rather than on erythroid cell proliferation. As the FV complex induces massive proliferation and terminal differentiation of erythroid progenitor cells in mice possessing the *Fv2^s* allele, the above-described increase in RAE-1 expression on TER-119^{Lo} cells may depend on the specific differentiation stage (proerythroblasts and early basophilic erythroblasts) or activated status of red cell precursors, rather than on FV infection. To examine this, we first induced an increased erythropoiesis by administering phenylhydrazine (PHZ) instead of FV infection. After administration of PHZ, both the TER-119^{Lo} and TER-119^{Hi} populations became discernible in the spleens of CB6F₁ mice; however, neither population in PHZ-treated mice showed increased expression of RAE-1, unlike the gp70⁺ TER-119^{Lo} population in FV-infected mice (Fig. 6A). Similar results were also obtained for MULT1. Thus, the

increase in RAE-1 and MULT1 expression on the TER-119^{Lo} population was likely caused by FV infection, not by induced erythropoiesis.

To further elucidate putative relationships between FV replication and RAE-1 expression on target cells of FV infection, mice deficient of either of the FV resistance factors APOBEC3 and BAFF-R, which show markedly enhanced FV replication and exaggerated pathology (46, 47), were examined. Mice of the *Fv2st* background were utilized so that the effects of FV replication on RAE-1 expression could be further separated from those of SFFV-induced erythroid cell proliferation. Both APOBEC3- and BAFF-R-deficient mice showed markedly increased numbers of gp70-expressing cells in the spleen in comparison with those in the wild-type (WT) animals (Fig. 6B). Importantly, RAE-1-positive cells increased in association with the increased numbers of gp70⁺ cells in APOBEC3- and BAFF-R-deficient animals. In APOBEC3-deficient mice, most of the gp70⁺ cells were TER-119⁺ and B220⁻, and these cells showed increased expression of RAE-1 proteins on their surfaces (Fig. 6B, arrows). In mice deficient of BAFF-R, even larger numbers of both TER-119⁺ (Fig. 6B, orange dots) and TER-119⁻ cells (Fig. 6B, red dots) were infected with FV, and these gp70⁺ cells expressed higher levels of RAE-1 than gp70⁻ cells. Interestingly, in FV-infected *BAFF-R*^{-/-} mice a significant proportion of B220⁺ B lymphocytes were also infected with FV, and they expressed higher levels of RAE-1 than uninfected B cells (Fig. 6B, blue dots).

As SFFV-induced growth potentiation of erythroid cells is limited in the above-described *Fv2^s* B6-background mice and as gp70⁺ B cells, as well as erythroid cells, expressed higher levels of RAE-1 proteins than gp70⁻ cells, the above results suggested that the replication of F-MuLV alone in the absence of SFFV might induce increased RAE-1 expression on target cells. Thus, we next infected the same gene-targeted animals with an infectious molecular clone of F-MuLV. In APOBEC3-

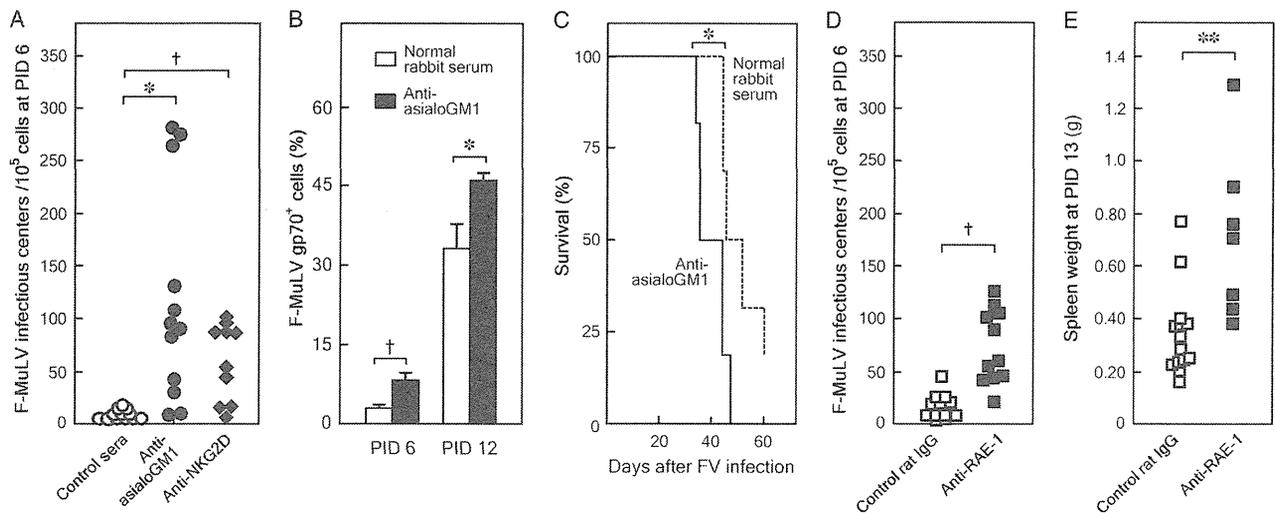


FIG. 5. Effects of the administration of anti-asialoGM1, anti-NKG2D, or anti-RAE-1 Ab on the expansion of virus-producing cells in the spleen and the development of FV-induced pathologies. (A) CB6F₁ mice were injected with either the anti-asialoGM1 or the blocking anti-NKG2D Ab and inoculated with 150 SFFU of FV. Control mice were injected with a mixture of normal rabbit and normal rat sera (control sera), and similarly infected. F-MuLV infectious centers in the spleen were enumerated by fluorescent focus assays as described previously (19, 44–47). Each symbol represents a datum from an individual mouse. As the anti-asialoGM1 and anti-NKG2D Ab-injected groups were compared with the common control group given the mixture of unimmunized sera, Bonferroni's correction was performed for multiple comparisons: *, $P = 0.0028 < \alpha(0.05) = 0.0253$; †, $P < 0.0005 < \alpha(0.05) = 0.0253$ by Welch's *t* test. (B) CB6F₁ mice were injected with the anti-asialoGM1 Ab as described previously (16) and inoculated with 150 SFFU of FV. Percentages of NK cells expressing both DX5 and NK-1.1 markers among spleen cells were monitored by multicolor flow cytometry as shown in Fig. 1, inset, and percentages of gp70⁺ cells were determined by the specific binding of Ab 720 at the indicated time points. Data shown here are mean \pm SEM calculated with values obtained from 5 to 12 animals per group at each time point. Comparisons were made between the anti-asialoGM1-treated and control groups: *, $P < 0.03$; †, $P < 0.005$ by Welch's *t* test. (C) Survival of anti-asialoGM1-treated animals ($n = 6$) after FV infection compared with that of control mice given normal rabbit serum ($n = 6$). As CB6F₁ mice are highly susceptible to FV and most died within 60 days after inoculation with as low as 15 SFFU in a previous experiment (16), a slightly lower dose of 50 SFFU was selected here in an attempt to detect the possibly increased susceptibility in NK-depleted mice. Two survival curves were compared by Mantel-Cox log-rank test: *, $P = 0.026$. (D and E) Groups of CB6F₁ mice were injected with either the RAE-1-blocking Ab (49) or control rat IgG and inoculated with 150 (D) or 50 SFFU (E) of FV. Spleen infectious centers were enumerated at PID 6 as described previously (19, 44–47), separate groups were killed at PID 13, and their spleen weights measured. The slightly reduced dose was utilized for the experiment shown in panel E, for the sake of consistency with the survival experiment described in the legend to panel C. Each symbol represents a datum from an individual mouse. †, $P < 0.0005$ by Welch's *t* test; **, $P = 0.0028$ by Student's *t* test.

deficient mice, a large proportion of TER-119⁺ cells were infected and expressed gp70; however, unlike the TER-119⁺ gp70⁺ cells in the same gene-targeted mice infected with FV complex, these cells showed only a slight increase in RAE-1 expression (Fig. 7A, arrows). Similar effects of the absence of the SFFV component on the expression of RAE-1 in erythroid cells were more evidently observed with BAFF-R-deficient mice: TER-119⁺ cells were infected with F-MuLV and expressed gp70, but unlike the same population of erythroid cells in FV-infected mice, these cells did not show a marked increase in the expression of RAE-1 proteins on their surfaces (Fig. 7A, orange dots). On the other hand, B220⁺ B cells in F-MuLV-infected BAFF-R^{-/-} mice (Fig. 7A, blue dots) showed increased expression of RAE-1 as observed upon FV infection.

To further confirm the above differences in RAE-1 expression levels between erythroid cells and B lymphocytes upon FV and F-MuLV infections, TER-119⁺ and B220⁺ cells were gated and their expression levels of RAE-1 proteins were compared between gp70⁺ and gp70⁻ populations. When infected with the FV complex, the gp70⁺ populations of both TER-119⁺ and B220⁺ cells expressed significantly higher levels of RAE-1 than the corresponding gp70⁻ cells in both APOBEC3- and BAFF-R-deficient animals (Fig. 7B). The average levels of

RAE-1 expression on gp70⁺ cells were 2.69 ± 0.12 and 3.28 ± 0.26 times higher than those on gp70⁻ cells among TER-119⁺ and B220⁺ cell populations, respectively, in FV-infected BAFF-R-deficient mice. On the other hand, although levels of RAE-1 expression between gp70⁺ and gp70⁻ populations were significantly different, gp70⁺ cells among TER-119⁺ erythroid cells in F-MuLV-infected BAFF-R-deficient mice expressed on their surfaces a level of RAE-1 proteins only 1.47 ± 0.06 times higher than that of gp70⁻ cells, while RAE-1 expression in the gp70⁺ population of B220⁺ B lymphocytes became even higher than that observed with FV-infected mice. In fact, the average level of RAE-1 expression on gp70⁺ cells among B220⁺ cells was 4.54 ± 0.13 times higher than that on gp70⁻ cells. Thus, these results indicate that SFFV is required for highly increased expression of RAE-1 on TER-119⁺ erythroid cells, while replication of F-MuLV alone induces increased RAE-1 expression in B220⁺ B cells.

DISCUSSION

As we have shown here, NKG2D⁺ NK cells expanded in the spleen and the levels of RAE-1 expression became preferentially higher on the surfaces of viral gp70-expressing cells in FV-infected mice. Although we have not tested all known

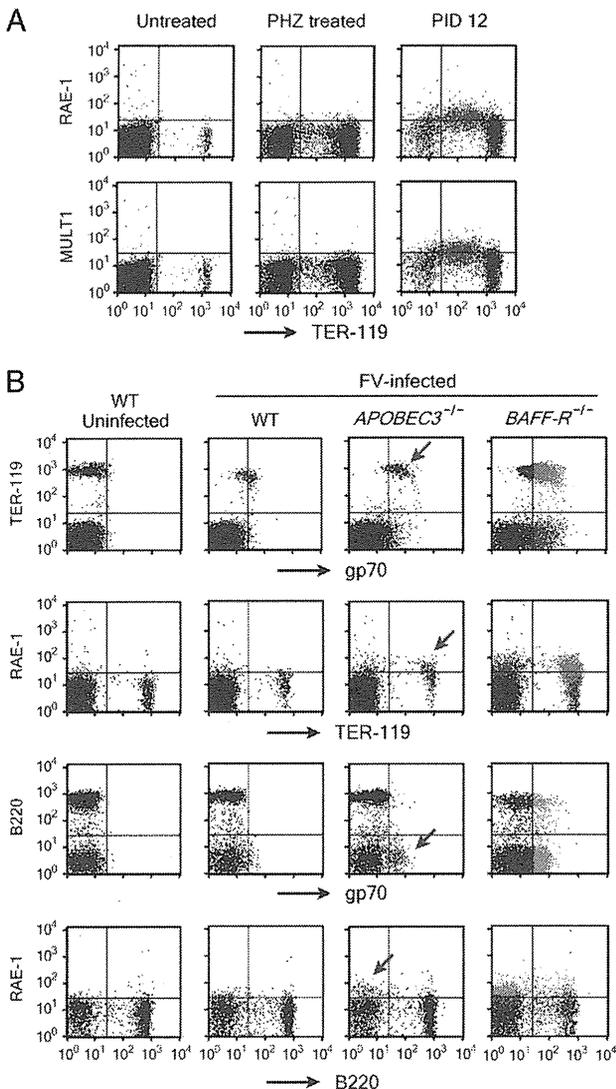


FIG. 6. RAE-1 expression on erythroid progenitor cells is induced by FV infection, not by erythropoiesis. (A) Representative dot plots showing the expression of NK receptor ligand proteins on the surfaces of nucleated spleen cells in phenylhydrazine-treated CB6F₁ mice. CB6F₁ mice were either injected with 1.2 mg/mouse PHZ on days 0 and 1 or inoculated with 150 SFFU FV on day 0, and at least 4 mice were killed on day 5 or day 12, respectively, to remove the spleen. Multicolor flow cytometric analyses were performed on nucleated cells with each indicated Ab. Demarcation lines were set based on the fluorescence levels observed by incubating the cells with isotype control Ab. Patterns observed with all 4 mice for each experimental group were consistent with those shown here. (B) Representative dot plots showing the expression of viral gp70, RAE-1, erythroid marker TER-119, and B-cell marker B220 on the surfaces of nucleated spleen cells in FV-infected B6 mice lacking an FV-resistance factor. As *Fv2^{tr}* mice were utilized, they were infected with 5×10^4 SFFU of FV complex, and 4 to 5 mice for each group were killed to remove the spleen. Flow cytometric analyses were performed as described in the legend to Fig. 3. Representative data obtained at PID 10 are shown here. In panel A, red dots, TER-119⁺ gp70^{hi} cells. In panel B, red dots, TER-119⁺ gp70⁺ cells; orange dots, TER-119⁺ gp70⁺ cells; purple dots, B220⁺ gp70⁺ cells; blue dots, B220⁺ gp70⁺ cells in FV-infected BAFF-R^{-/-} mice.

ligands for NK cell receptors, the nearly complete abrogation of the killing activities against F-MuLV-induced FBL-3 target cells exerted by DX5⁺ cells purified from FV-infected CB6F₁ mice *in vitro* and the significant increase in the number of FV infectious centers *in vivo* in the presence of the NKG2D-blocking Ab clearly indicate that the NKG2D receptor is involved primarily in the recognition and elimination of infected erythroid cells in FV-inoculated animals. Further, although the levels of induction of RAE-1 proteins on the surfaces of gp70⁺ erythroid cells were relatively low, as detectable by flow cytometry with the currently utilized Ab, and the same gp70⁺ TER-119^{Lo} cells retained Qa-1^b molecules on their surfaces, the induced RAE-1 proteins are apparently sufficient to induce the elimination of FV-infected erythroid cells *in vivo*, as the administration of the RAE-1-blocking Ab resulted in significantly increased numbers of infectious centers and more pronounced splenomegaly after FV inoculation. In this regard, FBL-3 cells were killed efficiently by purified NK cells despite their expression of Qa-1^b on the surfaces. Thus, NKG2D-RAE-1 interactions are involved in the elimination of virus-infected cells in the early stages of FV infection *in vivo*. These results are consistent with the previous finding that overexpression of VEGF-A paradoxically resulted in delayed development of FV-induced leukemia, probably due partly to enhanced NK cell activities in the transgenic mice (4).

The mechanisms by which FV infection upregulates the expression of RAE-1 molecules in erythroid progenitor cells and B lymphocytes are currently unknown. Several cytokines, including IFN- γ , have been shown to upregulate the expression of these retinoid-inducible genes (8, 49), and we have demonstrated in the present study that IFN- γ augments the expression of *Rae1* genes in F-MuLV-induced leukemia cells. As the production of IFN- γ in the spleens of FV-infected mice has been detected as early as 5 days after virus inoculation (31), the induction of *Rae1* gene expression in FV-infected cells might be mediated by IFN- γ . However, as higher levels of RAE-1 proteins were expressed preferentially on gp70⁺ cells, it is unlikely that cytokine-mediated induction, which should work on both FV-infected and uninfected cells in the spleen, is responsible mainly for the increased levels of RAE-1 protein expression on FV-infected cells. The lack of increased RAE-1 expression on TER-119^{Lo} cells in PHZ-injected mice also indicates the presence of a virus-specific, rather than a differentiation- or growth-associated, mechanism of RAE-1 induction. In this regard, infection of mouse primary B lymphocytes with highly leukemogenic Abelson MuLV induces the expression of activation-induced cytidine deaminase (AID), and the genotoxic action of AID leads to upregulation of NKG2D ligands on infected cell surfaces (11). HIV-1 Vpr is also known to upregulate the expression of NKG2D ligands on cell surfaces (35), and the above Vpr-induced upregulation of NKG2D ligand expression has been shown to be mediated through the DNA damage response (48). Thus, it is tempting to speculate that repeated proviral integrations into the erythroid progenitor cells that are facilitated in the presence of SFFV-induced growth potentiation may cause DNA damage and the resultant upregulation of RAE-1 expression in FV-infected erythroid cells.

Taking advantage of known FV-resistant host factors (5, 12, 25, 27, 46, 47), we successfully dissected here the effect of

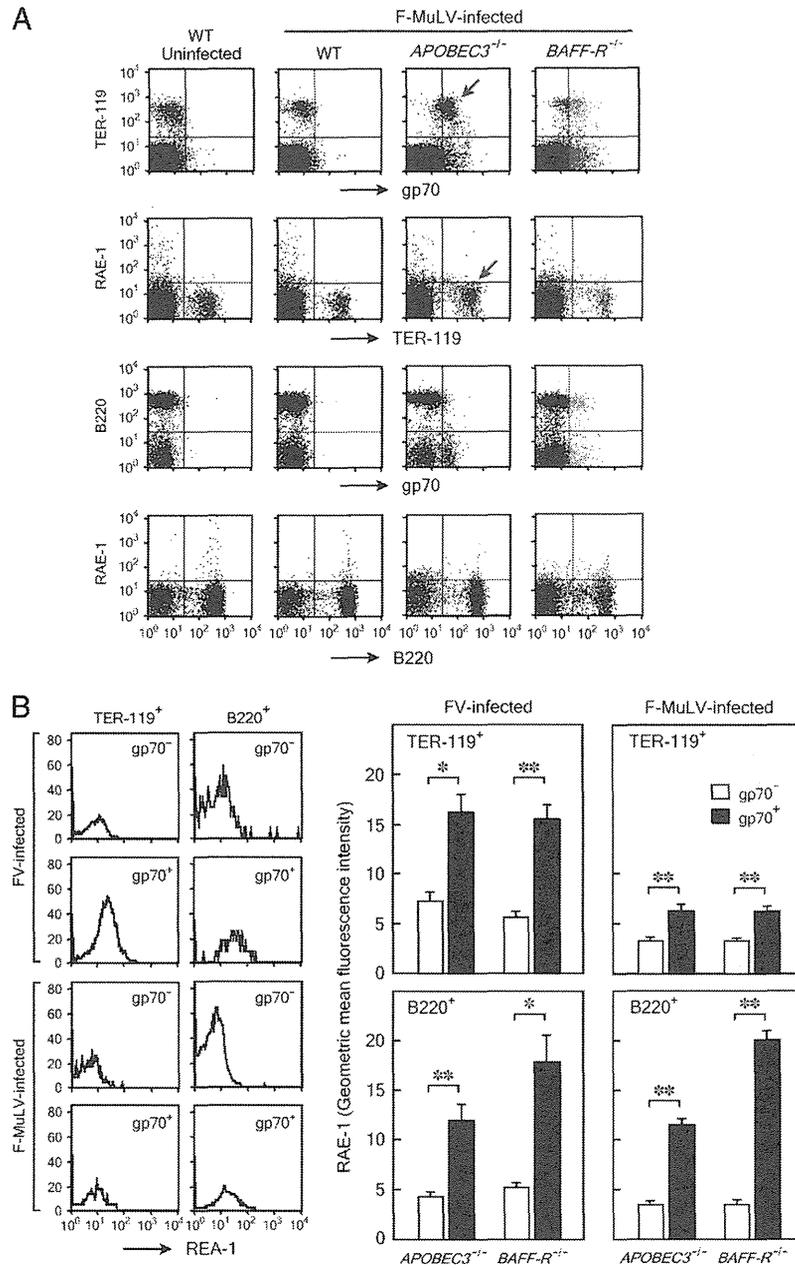


FIG. 7. RAE-1 expression on spleen cells upon infection with F-MuLV alone. (A) Representative dot plots showing the expression of viral gp70, RAE-1 proteins, TER-119, and B220 on the surfaces of nucleated spleen cells in F-MuLV-infected B6 mice lacking an FV resistance factor. Mice were infected with 5×10^4 fluorescent focus units of F-MuLV (FB-29), and 4 or 5 mice for each group were killed to remove the spleen. Flow cytometric analyses were performed as described for Fig. 3. Representative data obtained at PID 10 are shown here. Arrows, slight increase in RAE-1 expression on the surfaces of TER-119⁺ gp70⁺ cells in F-MuLV-infected *APOBEC3*^{-/-} mice. Red dots, TER-119⁻ gp70⁺ cells; orange dots, TER-119⁺ gp70⁻ cells; purple dots, B220⁻ gp70⁺ cells; blue dots, B220⁺ gp70⁺ cells in F-MuLV-infected *BAFF-R*^{-/-} mice. (B) Representative histograms on the left show different levels of RAE-1 expression on gp70⁻ and gp70⁺ populations of TER-119⁺ erythroid and B220⁺ B cells. Averages of GMFI for RAE-1 compared between the gp70⁻ and gp70⁺ populations of TER-119⁺ erythroid cells and B220⁺ B lymphocytes are shown on the right. Each bar shows mean \pm SEM calculated with GMFI data obtained from 4 or 5 mice. *, $P < 0.02$; **, $P < 0.004$ by paired t test.

SFFV-induced erythroid cell growth potentiation from that of FV replication on RAE-1 induction in target cells. In fact, FV infection in the absence of massive erythroid cell proliferation still resulted in highly increased RAE-1 expression on TER-

119⁺ erythroid cells in *Fv2*⁻ B6 mice lacking either *APOBEC3* or *BAFF-R*. Highly increased expression of RAE-1 on infected erythroid as well as B cells in the gene-targeted B6 mice indicates a close correlation between high levels of retroviral rep-

lication and induction of RAE-1 expression on target cells. It should be noted, however, that although B cells expressed similarly increased levels of RAE-1 proteins upon infection with FV complex or with F-MuLV alone, levels of RAE-1 induction on erythroid cells became much higher in the presence of the SFFV component. Thus, in the above conditions of nonrestricted retroviral replication, F-MuLV alone can induce RAE-1 expression in B cells, while SFFV is also required for the induction of high levels of RAE-1 in erythroid cells. In fact, SFFV-induced growth potentiation of infected erythroid cells in the *Fv2^s*-possessing CB6F₁ mice further enhanced RAE-1 expression on gp70⁺ erythroblasts, as RAE-1 levels on gp70⁺ cells in CB6F₁ mice were apparently higher than those in the gene-targeted B6 mice (compare fluorescence intensities between Fig. 4 and 7). Nevertheless, the above cell type-associated differences in RAE-1 expression in F-MuLV-infected B6 mice may facilitate further dissection of viral and cellular factors that are involved in retrovirus-induced RAE-1 upregulation.

In the present study, we observed a lag in the increase of TER-119⁺ erythroblasts until PID 8, followed by an abrupt and massive increase of gp70⁺ cells in the spleen by PID 12. An abrupt increase in the number of TER-119⁺ cells in the spleen starting around PID 8 has repeatedly been observed with *Fv2^s*-possessing mice (16, 19, 26), which is consistent with a recent finding of two distinctive populations of target cells for FV infection (43). Thus, one population expresses the short-form STK and generates erythropoietin-independent erythroid burst-forming units (BFU-E) in the bone marrow soon after FV infection, while cells of the other population migrate into the spleen as infectious centers, interact with the stromal cells to express bone morphogenic protein 4, and cause the expansion of stress BFU-E. It is tempting to speculate that the cells expressing increased levels of RAE-1 and MULT1 are FV-infected stress BFU-E and that they are susceptible to NK killing. As the depletion of NK cells or the blockade of either NKG2D or RAE-1 resulted in significantly increased FV infectious centers in the spleen at as early as PID 6, followed by significantly more pronounced splenomegaly and earlier death, it is also possible that NK cells are restricting the migration of CD31⁺ Kit⁺ CD41⁺ Sca1⁻ Lin⁻ infectious center cells into the spleen. In fact, immunization of mice with the gp70-derived Th-cell epitope suppressed the above abrupt expansion of TER-119⁺ cells in the spleen that otherwise started from PID 8 (16, 19, and 26), and this vaccine effect was abrogated by depletion of NK cells (16), indicating that Th cell-mediated activation of NK cells may have suppressed the migration of FV infectious centers from bone marrow. Thus, this model may also shed light on basic mechanisms through which the migration of committed erythroid progenitor cells from bone marrow to the spleen might be regulated by NK cell functions.

ACKNOWLEDGMENTS

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, including the High-Tech Research Center, Medico-Technical Cooperation, and Anti-Aging Center grants, those from the Ministry of Health, Labor, and Welfare of Japan for Research on HIV/AIDS, and those from the Japan Health Sciences Foundation. The use of radioisotopes, animal experiments, and flow cytometric analyses were sup-

ported by members of the Central Research Facilities, Kinki University School of Medicine.

We are grateful to J. Brian Dowell for critically reading and correcting the manuscript and to Yoshiko Ito, Department of Respiratory Medicine and Allergology, Kinki University School of Medicine, for her help in operating the AutoMACS.

REFERENCES

- Alter, G., et al. 2007. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J. Infect. Dis.* **195**:1452–1460.
- Arase, H., T. Saito, J. H. Phillips, and L. L. Lanier. 2001. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (α_2 integrin, very late antigen-2). *J. Immunol.* **167**:1141–1144.
- Azakami, K., et al. 2009. Severe loss of invariant NKT cells exhibiting anti-HTLV-1 activity in patients with HTLV-1-associated disorders. *Blood* **114**:3208–3215.
- Cervi, D., et al. 2007. Enhanced natural-killer cell and erythropoietic activities in VEGF-A-overexpressing mice delay F-MuLV-induced erythroleukemia. *Blood* **109**:2139–2146.
- Chesebro, B., M. Miyazawa, and W. J. Britt. 1990. Host genetic control of spontaneous and induced immunity to Friend murine retrovirus infection. *Annu. Rev. Immunol.* **8**:477–499.
- Costello, R. T., C. Fauriat, S. Sinori, E. Marcenaro, and D. Olive. 2004. NK cells: innate immunity against hematopoietic malignancies? *Trends Immunol.* **26**:328–333.
- Dittmer, U., et al. 2001. Role of interleukin-4 (IL-4), IL-12, and gamma interferon in primary and vaccine-primed immune responses to Friend retroviral infection. *J. Virol.* **75**:654–660.
- Faria, P. A., et al. 2005. VSV disrupts the Rael/mrnp41 mRNA nuclear export pathway. *Mol. Cell* **17**:93–102.
- Gaudieri, S., et al. 2005. Killer immunoglobulin-like receptors and HLA act both independently and synergistically to modify HIV disease progression. *Genes Immun.* **6**:683–690.
- Giavedoni, L. D., M. C. Velasquillo, L. M. Parodi, G. B. Hubbard, and V. L. Hodora. 2000. Cytokine expression, natural killer cell activation, and phenotypic changes in lymphoid cells from rhesus macaques during acute infection with pathogenic simian immunodeficiency virus. *J. Virol.* **74**:1648–1657.
- Gourzi, P., T. Leonova, and F. N. Papavasiliou. 2006. A role for activation-induced cytidine deaminase in the host response against a transforming retrovirus. *Immunity* **24**:779–786.
- Hasenkrug, K. J., and B. Chesebro. 1997. Immunity to retroviral infection: the Friend virus model. *Proc. Natl. Acad. Sci. U. S. A.* **94**:7811–7816.
- Ho, E. L., et al. 2002. Costimulation of multiple NK cell activation receptors by NKG2D. *J. Immunol.* **169**:3667–3675.
- Howcroft, T. K., and D. S. Singer. 2003. Expression of nonclassical MHC class II genes: comparison of regulatory elements. *Immunol. Res.* **27**:1–30.
- Iannello, A., O. Debeche, S. Samarani, and A. Ahmad. 2008. Antiviral NK cell responses in HIV infection. I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS. *J. Leuko. Biol.* **84**:1–26.
- Iwanami, N., A. Niwa, Y. Yasutomi, N. Tabata, and M. Miyazawa. 2001. Role of natural killer cells in resistance against Friend retrovirus-induced leukemia. *J. Virol.* **75**:3152–3163.
- Jamieson, A. M., et al. 2002. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17**:19–29.
- Kabat, D. 1989. Molecular biology of Friend viral erythroleukemia. *Curr. Top. Microbiol. Immunol.* **148**:1–42.
- Kawabata, H., et al. 2006. Peptide-induced immune protection of CD8⁺ T cell-deficient mice against Friend retrovirus-induced disease. *Int. Immunol.* **18**:183–198.
- Kina, T., et al. 2000. The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *Br. J. Haematol.* **109**:280–287.
- Kottihil, S., et al. 2003. Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *J. Infect. Dis.* **187**:1038–1045.
- Maier, L. M., et al. 2008. NKG2D-RAE-1 receptor-ligand variation does not account for the NK cell defect in Nonobese diabetic mice. *J. Immunol.* **181**:7073–7080.
- Martin, M. P., et al. 2002. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat. Genet.* **31**:429–434.
- Martin, M. P., et al. 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat. Genet.* **39**:733–740.
- Miyazawa, M. 2004. Host genes that influence immune and non-immune resistance mechanisms against retroviral infections. *Rec. Res. Dev. Virol.* **6**:105–118.
- Miyazawa, M., et al. 1995. Immunization with a single T helper cell epitope abrogates Friend virus-induced early erythroid proliferation and prevents late leukemia development. *J. Immunol.* **155**:748–758.
- Miyazawa, M., S. Tsuji-Kawahara, and Y. Kanari. 2008. Host genetic factors that control immune responses to retrovirus infections. *Vaccine* **26**:2981–2996.

28. Ney, P. A., and A. D. D'Andrea. 2000. Friend erythroleukemia revisited. *Blood* **96**:3675–3680.
29. Ogasawara, K., et al. 2004. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* **20**:757–767.
30. Ogasawara, K., and L. L. Lanier. 2005. NKG2D in NK and T cell-mediated immunity. *J. Clin. Immunol.* **25**:534–540.
31. Peterson, K. E., M. Iwashiro, K. J. Hasenkrug, and B. Chesebro. 2000. Major histocompatibility complex class I gene controls the generation of gamma interferon-producing CD4⁺ and CD8⁺ T cells important for recovery from Friend retrovirus-induced leukemia. *J. Virol.* **74**:5363–5367.
32. Peterson, K. E., I. Stromnes, R. Messer, K. Hasenkrug, and B. Chesebro. 2002. Novel role of CD8⁺ T cells and major histocompatibility complex class I genes in the generation of protective CD4⁺ Th1 responses during retrovirus infection in mice. *J. Virol.* **76**:7942–7948.
33. Pradet-Balade, B., C. Leberbauer, N. Schweifer, and F. Boulmé. 2010. Massive translational repression of gene expression during mouse erythroid differentiation. *Biochim. Biophys. Acta* **1799**:630–641.
34. Ravet, S., et al. 2007. Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals. *Blood* **109**:4296–4305.
35. Richard, J., S. Sindhu, T. N. Pham, J. P. Belzile, and E. A. Cohen. 2010. HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. *Blood* **115**:1354–1363.
36. Robertson, M. N., et al. 1991. Production of monoclonal antibodies reactive with a denatured form of the Friend murine leukemia virus gp70 envelope protein: use in a focal infectivity assay, immunohistochemical studies, electron microscopy and Western blotting. *J. Virol. Methods* **34**:255–271.
37. Sasaki, Y., S. Casola, J. L. Kutok, K. Rajewsky, and M. Schmidt-Supprian. 2004. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *J. Immunol.* **173**:2245–2252.
38. Scott-Algara, D., et al. 2003. Increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravenous drug users. *J. Immunol.* **171**:5663–5667.
39. Seaman, W. E. 2000. Natural killer cells and natural killer T cells. *Arthritis Rheum.* **43**:1204–1217.
40. Sieff, C., et al. 1982. Changes in cell surface antigen expression during hemopoietic differentiation. *Blood* **60**:703–713.
41. Slifka, M. K., R. R. Pagarigan, and J. L. Whitton. 2000. NK markers are expressed on a high percentage of virus-specific CD8⁺ and CD4⁺ T cells. *J. Immunol.* **164**:2009–2015.
42. Stromnes, I. M., et al. 2002. Temporal effects of gamma interferon deficiency on the course of Friend retrovirus infection in mice. *J. Virol.* **76**:2225–2232.
43. Subramanian, A., et al. 2008. Friend virus utilizes the BMP4-dependent stress erythropoiesis pathway to induce erythroleukemia. *J. Virol.* **82**:382–393.
44. Sugahara, D., S. Tsuji-Kawahara, and M. Miyazawa. 2004. Identification of a protective CD4⁺ T-cell epitope in p15^{FR} of Friend murine leukemia virus and role of the MA protein targeting the plasma membrane in immunogenicity. *J. Virol.* **78**:6322–6334.
45. Takamura, S., et al. 2010. Premature terminal exhaustion of Friend virus-specific effector CD8⁺ T cells by rapid induction of multiple inhibitory receptors. *J. Immunol.* **184**:4696–4707.
46. Takeda, E., et al. 2008. Mouse APOBEC3 restricts Friend leukemia virus infection and pathogenesis in vivo. *J. Virol.* **82**:10998–11008.
47. Tsuji-Kawahara, S., et al. 2010. Persistence of viremia and production of neutralizing antibodies differentially regulated by polymorphic APOBEC3 and BAFF-R loci in Friend virus-infected mice. *J. Virol.* **84**:6082–6095.
48. Ward, J., et al. 2009. HIV-1 Vpr triggers natural killer cell-mediated lysis of infected cells through activation of the ATR-mediated DNA damage response. *PLoS Pathog.* **5**:e1000613.
49. Yokoyama, W. M., and B. F. M. Plougastel. 2003. Immune functions encoded by the natural killer gene complex. *Nat. Rev. Immunol.* **3**:304–316.
50. Zhang, J., M. Socolovsky, A. W. Gross, and H. F. Lodish. 2003. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood* **102**:3938–3946.
51. Zhou, R., H. Wei, R. Sun, J. Zhang, and Z. Tian. 2007. NKG2D recognition mediates Toll-like receptor 3 signaling-induced breakdown of epithelial homeostasis in the small intestines of mice. *Proc. Natl. Acad. Sci. U. S. A.* **104**:7512–7515.

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^{1,9}, Akira Maruyama¹, Misako Matsumoto¹, Hideo Yagita², Tsukasa Seiya^{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

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)

⁹ 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 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- β 1 to induce Foxp3 [27,28]. However, Pam2 lipopeptides did not up-regulate the mRNA of TGF- β 1 (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 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

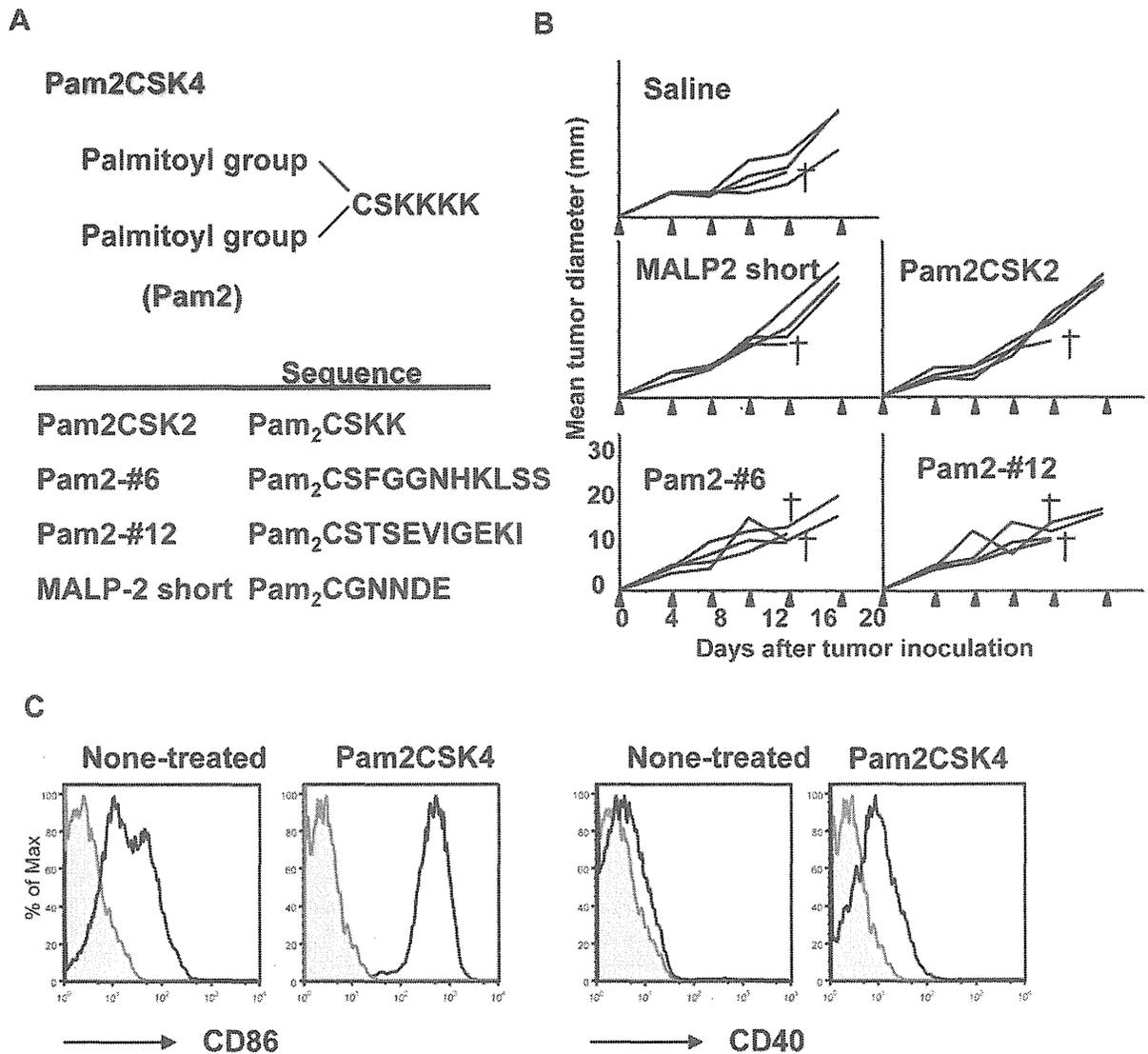


Figure 1. Pam2 lipopeptides do not induce effective anti-tumor immunity. (A) Structures of the Pam2 lipopeptides are shown. (B) Mice were injected with B16D8 melanoma cells (2×10^5) on back. The mice were injected s.c. into their footpad with the indicated Pam2 lipopeptides (10 nmol) or saline twice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

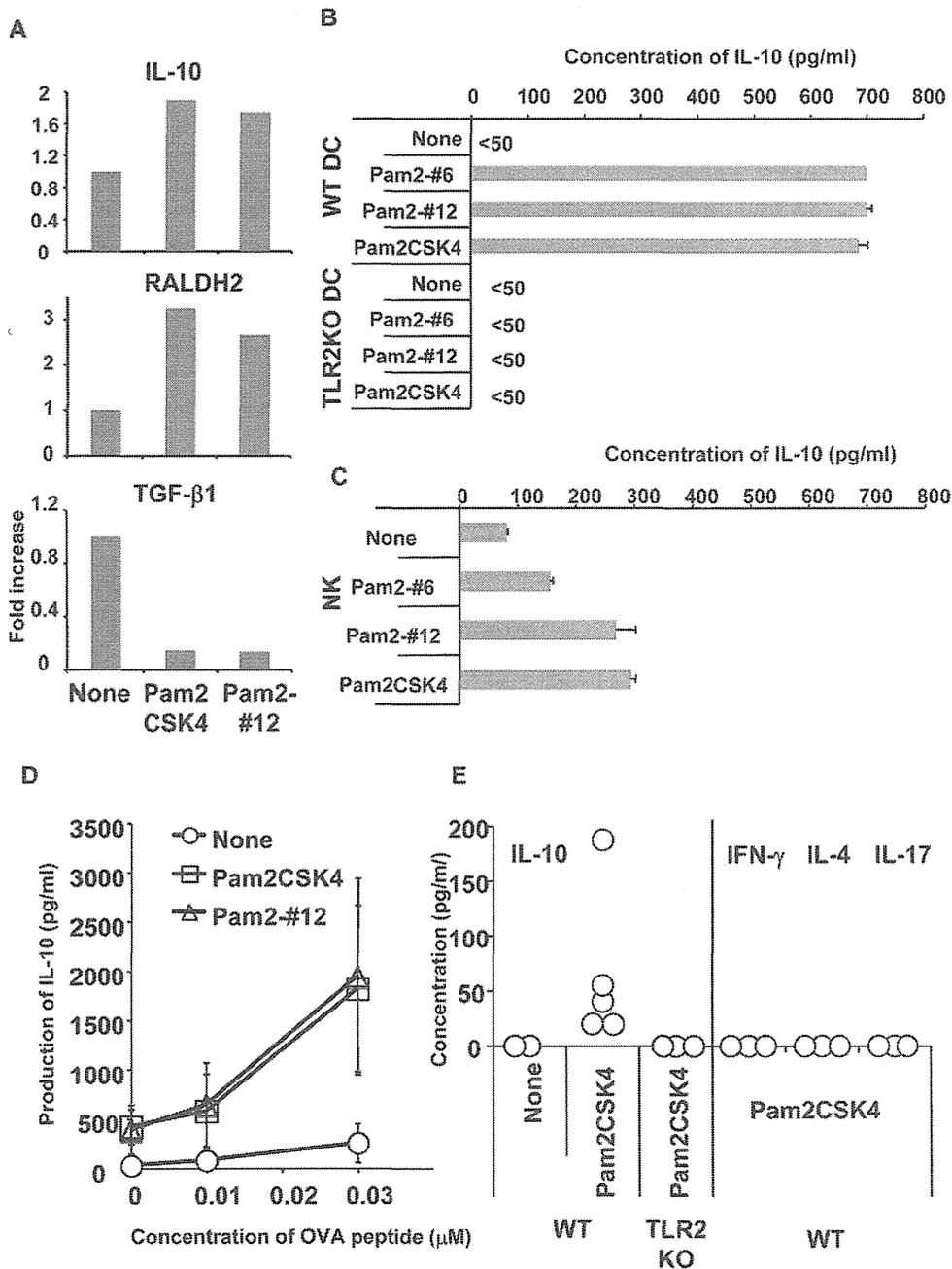


Figure 2. Pam2 lipopeptides induce IL-10 and retinal dehydrogenase. (A) Spleen DCs from B6 mice were cultured with or without 100 nM of Pam2CSK4 or Pam2-#12. After four hours, total RNA was prepared and real-time PCR was performed. Expression of each sample was normalized to GAPDH mRNA expression and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

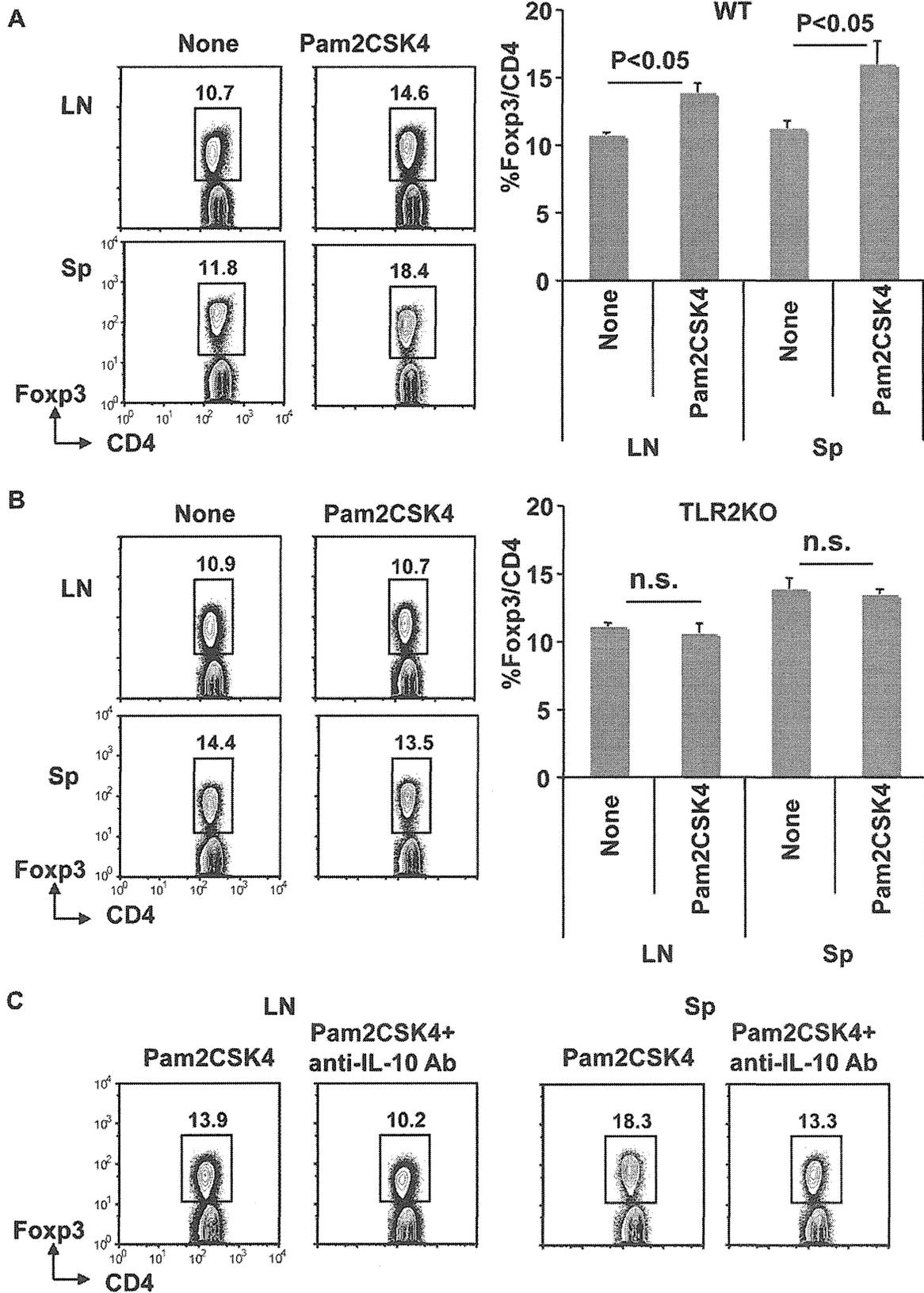


Figure 3. Systemic injection of Pam2CSK4 expands Foxp3⁺ T reg cells in a TLR2- and IL-10-dependent manner. (A) WT mice were i.p. injected with Pam2CSK4 (10 nmol). After three days, spleen (Sp) and lymph node (LN) cells were analyzed for the expression of Foxp3. The plots were gated on CD4⁺ T cells. One 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⁺ 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 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 naive 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.

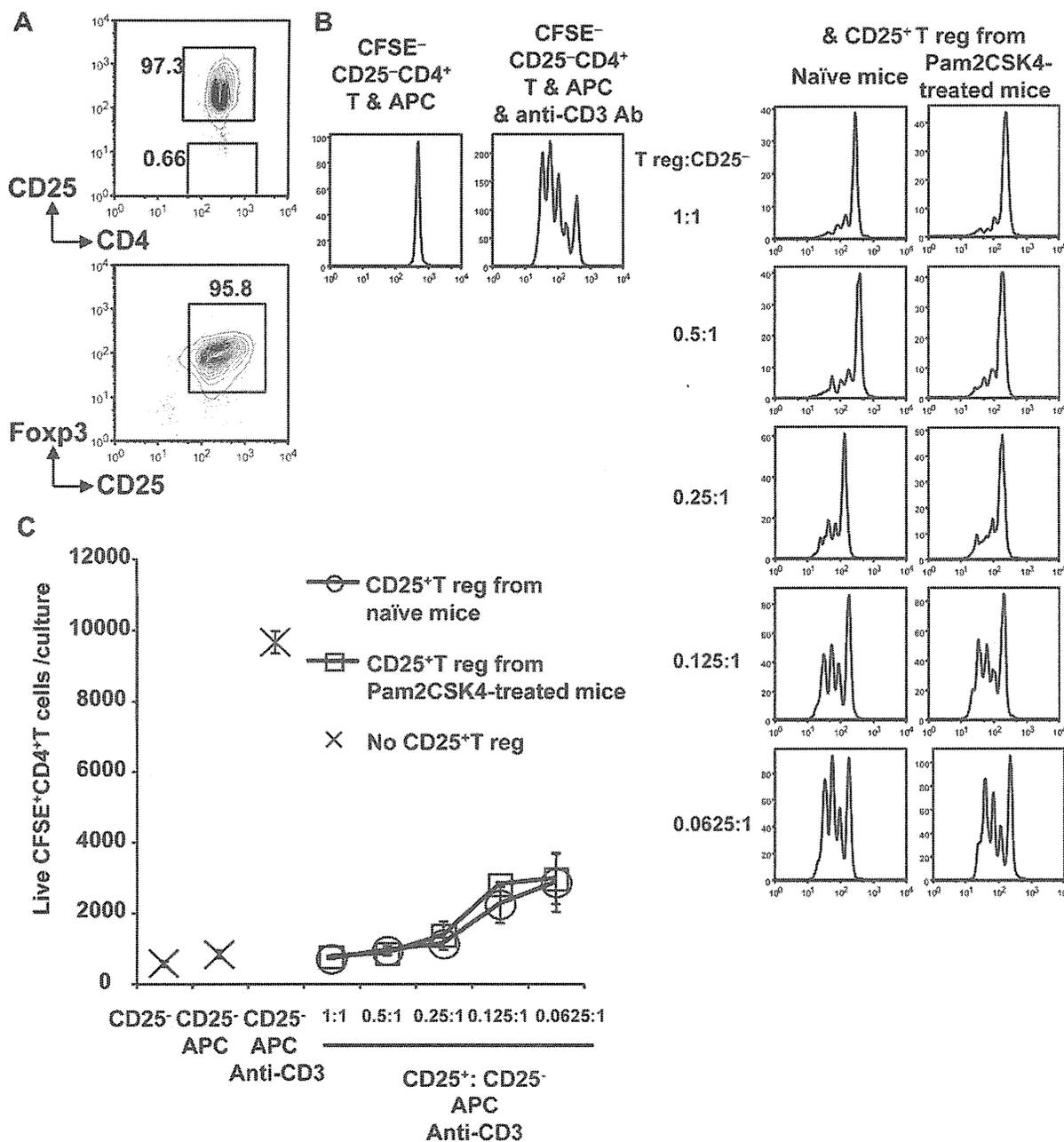


Figure 4. T reg cells from Pam2CSK4-treated mice maintain suppressive activity. (A) CD25⁺CD4⁺ T cells purified by flow cytometry were further fixed and stained with Foxp3. FACS plots were gated on CD4⁺ T cells. One of three similar experiments is shown. (B) B6 mice were i.p. injected with Pam2CSK4 (10 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 (5×10^4) were stimulated with irradiated spleen antigen presenting cells (1×10^5) 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

Materials and Methods

Mice

C57BL6J (B6) mice and CBI7SCID 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

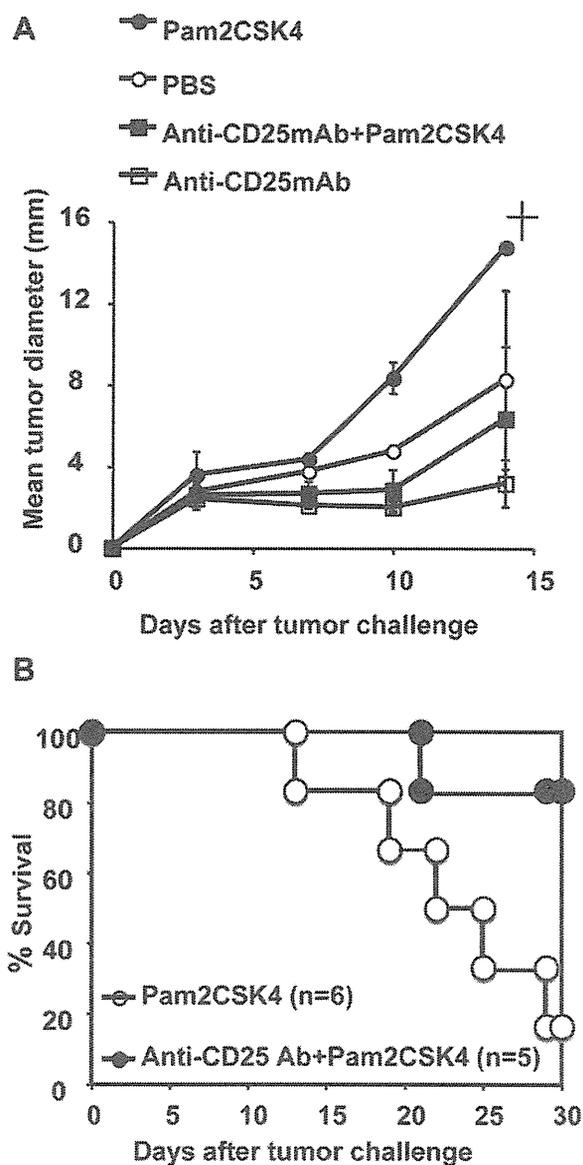


Figure 5. NK-sensitive tumors grow slowly when Pam2CSK4 is injected into T reg-depleted mice. (A) B6 mice were i.p. injected with 500 μ g of anti-CD25mAb (PC61) on day -3. The mice were injected with B16D8 melanoma cells (2×10^5) into their back on day 0. Pam2CSK4 (10 nmol) or PBS was injected twice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

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.

References

- Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. *Int J Cancer* 127: 759–767.
- Curiel TJ (2008) Regulatory T cells and treatment of cancer. *Curr Opin Immunol* 20: 241–246.
- 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.
- Li X, Kostareli E, Saffner J, Garbi N, Hammerling GJ (2010) Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. *Eur J Immunol* 40: 3325–3335.
- 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.
- 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.
- 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.
- 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.
- 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.
- Sutmoller RP, van Duivenvoorde LM, van Elsland 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.
- 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.
- 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.
- 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.
- Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. *Nature* 449: 419–426.
- 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.
- Dubensky TW, Jr., Reed SG (2010) Adjuvants for cancer vaccines. *Semin Immunol* 22: 155–161.
- Seya T, Matsumoto M (2009) The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. *Cancer Immunol Immunother* 58: 1175–1184.

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.

- 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.
- 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.
- 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.
- Azuma I, Seya T (2001) Development of immunoadjuvants for immunotherapy of cancer. *Int Immunopharmacol* 1: 1249–1259.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Yamazaki S, Bonito AJ, Spisek R, Dhodapkar M, Inaba K, et al. (2007) Dendritic cells are specialized accessory cells along with TGF-beta 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. Sattmuller RP, den Brok MH, Kramer M, Bennis 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.

Natural Killer Cell Activation Secondary to Innate Pattern Sensing

Tsukasa Seya Jun Kasamatsu Masahiro Azuma Hiroaki Shime
Misako Matsumoto

Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Key Words

Natural killer cell activation • Dendritic cell • Toll-like receptor 3 • TICAM-1 • MyD88

Abstract

Recent progress in understanding the outcomes of pattern-recognition by myeloid dendritic cells (mDC) allows us to delineate the pathways driving natural killer (NK) cell activation. Mouse mDC mature in response to microbial patterns and are converted to an NK cell-activating phenotype. The MyD88 pathway, the Toll/IL-1 receptor homology domain-containing adaptor molecule (TICAM)-1 (TRIF) pathway, and the interferon (IFN)- β promoter stimulator 1 (IPS-1) pathway in mDC participate in driving NK activation, as shown by analyses in knockout mice. Studies using synthetic compounds for Toll-like receptors/RIG-I-like receptors have demonstrated that mDC-NK cell contact induces NK cell activation without the participation of cytokines in mice. In vivo bone marrow transplantation analysis revealed that the IPS-1 pathway in nonmyeloid cells and the TICAM-1 pathway in mDC are crucial for dsRNA-mediated in vivo NK activation. These results infer the presence of cytokine-dependent and cytokine-independent modes of NK activation in conjunction with innate immune activation. Here, we focus on the IFN-inducing pathways and mDC-NK contact-induced NK activation and discuss the reported various NK activation modes.

Copyright © 2011 S. Karger AG, Basel

Introduction

Natural killer (NK) cells have the ability to kill certain tumor cells and infected cells [1]. A number of activating and inhibitory receptors have been implicated in NK cell recognition and elimination of target cells [2]. In addition, NK cell effector functions are induced or potentiated through recognition of microbial products by innate pattern recognition receptors (PRRs) that are expressed in various cell types, including myeloid cells and NK cells. In this way, dendritic cells may induce cytokines, such as interferon (IFN)- γ , and potentiate cytotoxicity by NK cells [3]. In mice, myeloid cells stimulate NK cells through cell-cell contact and with soluble mediators [4, 5]. Many factors including cytokines and molecules supporting direct contact by immune-related cells are reported to participate in NK activation [2–5]. Myeloid dendritic cells (mDC) and macrophages (M ϕ) often serve as a source of such activating factors as IL-12p70, IL-18, IL-15, and type I IFN.

It is well known that type I IFN activates NK cells. However, immature mDC only subfunctionally produce type I IFN and, consequently, IFN-dependent NK activation factors are maintained at basal levels through the IFN- α receptor (IFNAR) pathway in mice [6]. Infection or inflammation stimulates additional factors that render NK activation by mDC feasible [4, 5]. Such factors that induce mDC maturation largely belong to a class of ex-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2011 S. Karger AG, Basel
1662–811X/11/0033–0264\$38.00/0

Accessible online at:
www.karger.com/jin

Dr. Tsukasa Seya
Department of Microbiology and Immunology
Graduate School of Medicine, Hokkaido University
Kita-ku, Sapporo 060-8638 (Japan)
Tel. +81 11 706 5073, E-Mail seya-tu@pop.med.hokudai.ac.jp

ogenous or endogenous pattern molecules designated pathogen-associated molecular patterns (PAMP)/damage-associated molecular patterns (DAMP) [7, 8]. Research into signaling pathways in the innate immune system has indicated that PAMP and DAMP act on PRRs in mDC/Mf and drive NK activation [4, 5, 7, 8]. In addition, membrane molecules upregulated on the surface of mDC participate in NK activation in a process known as mDC-NK contact-mediated NK activation [4, 5]. In this case, mature mDC and NK cells must be recruited to local lymph nodes, and their interactions lead to the emergence of effector NK cells in the periphery. However, it is unknown whether activation of NK cells is totally dependent on IFN or just shares the PRR pathways with IFN induction to upregulate other NK-activating molecules. Furthermore, it remains undetermined what microenvironment mDC require for maturation along with NK activation and what effectors mDC stimulate via the PRR pathways to participate in enhancing NK activity.

Recent progress in the innate pattern-sensing system suggests that mDC pattern recognition is a major event in driving mDC to an NK-activating phenotype [9–11]. These results add new insight into the currently accepted theory that the balance between a number of activating receptors and inhibitory receptors and their activation states are critical for NK activation [2, 3, 12, 13]. Insight into the mechanism behind NK cell activation may be gained via analysis of the molecular mechanisms by which PAMP/DAMP activate the immune system and, in particular, mDC. NK cells have the capacity to induce memory-like responses in a way comparable to T lymphocytes [14], and some subsets are specialized to produce the Th17 cytokine IL-22 [15] although their features are not always comparable to NK cells. These unique features of NK cells may be associated with mDC factors that drive NK activation, including the combination of stimuli required for PRR and the cytokines that act in conjunction with inhibitory/activating ligands on NK receptors [16]. This review collates recent advances in the innate molecules and pathways related to mDC-mediated NK activation.

Direct or Secondary Activation of NK Cells by Microbial Patterns

DC/Mf as well as stromal cells express a variety of PRRs. In infection, these non-NK cell-derived mediators play a role in NK cell responses to pathogens [17]. Activation of these cells in response to PAMP can also lead to indirect NK activation which is mediated by affected accessory

cells with altered membrane-associated molecules [16, 17]. Both soluble factors and membrane molecules join NK cell activation. Several reviews mention the mode of direct and accessory cell-derived (secondary) NK activation [2, 8, 16]. We just summarized this issue to facilitate the introduction of mDC/Mf-mediated NK cell activation.

Pathogen molecules often interact with NK receptors. Examples of direct pathogen interaction with NK cells have been demonstrated in mouse cytomegalovirus, whose m157 molecule interacts with Ly49H, an NK cell-activating/inhibitory receptor [18]. Influenza virus hemagglutinins bind the NKP46 of human NK cells [19]. Besides these NK receptor-interacting molecules, several kinds of bacteria/viruses are known to directly activate NK cells by PRR stimulation. Examples of microbial ligands for Toll-like receptors (TLRs) present in the NK cell membrane are as follows. Measles virus H protein interacts with TLR2 [20]. Mycobacteria muramyl dipeptides activate TLR2 residing on the NK cell membrane [21]. *Plasmodium falciparum* has an unidentified factor that interacts with TLR2/4 [22]. Some leishmania species have a lipophosphoglycan to bind to TLR2 [23]. Pam2 lipopeptides of a variety of bacteria serve as TLR2 ligands [24]. These factors also interact with mDC/Mf TLRs. Which TLRs in NK cells or accessory cells are more important for triggering NK cell activation in vivo should be an issue to be clarified.

Overview of the mDC Pattern Sensing System

mDC, which comprise many subsets [including bone marrow-derived DC (BMDC) and CD8 α + DC], possess subset-specific pattern-recognition systems. TLR, NOD-like receptors, and RIG-I-like receptors (RLR) are representative PRRs. The PRR repertoire in mDC has been described previously [17]. Two adaptors, i.e. MyD88 and TICAM-1 (TRIF), critically determine the signal pathways for TLRs, whereas interferon- β promoter stimulator 1 (IPS-1) (Cardif, MAVS, VISA) is the only adaptor that governs MDA5/RIG-I signaling (fig. 1). These adaptors are engaged in type I IFN induction and NK activation via partly overlapped but distinct pathways in a cell type-specific manner. Here, we summarize the signal pathways for TLR and RLR relevant to NK activation.

Signaling Pathways That Operate through the MyD88 Adaptor

MyD88 is the most common adaptor in the TLR and interleukin (IL)-1R signaling pathways [25, 26]. With the