NKT cell–mediated repression of tumor immunosurveillance by IL-13 and the IL-4R–STAT6 pathway

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Using a mouse model in which tumors show a growth-regression-recurrence pattern, we investigated the mechanisms for down-regulation of cytotoxic T lymphocyte–mediated tumor immunosurveillance. We found that interleukin 4 receptor (IL-4R) knockout and downstream signal transducer and activator of transcription 6 (STAT6) knockout, but not IL-4 knockout, mice resisted tumor recurrence, which implicated IL-13, the only other cytokine that uses the IL-4R–STAT6 pathway. We confirmed this by IL-13 inhibitor (sIL-13Rα2–Fc) treatment. Loss of natural killer T cells (NKT cells) in CD1 knockout mice resulted in decreased IL-13 production and resistance to recurrence. Thus, NKT cells and IL-13, possibly produced by NKT cells and signaling through the IL-4R–STAT6 pathway, are necessary for down-regulation of tumor immunosurveillance. IL-13 inhibitors may prove to be a useful tool in cancer immunotherapy.

Many subclinical tumors expressing immunogenic tumor antigens spontaneously regress; only those that escape immunosurveillance reach the stage at which they are detected clinically. Although there have been many suggestions regarding mechanisms by which tumors escape from immunosurveillance[1–4], the absence of an animal tumor model in which tumors recur after spontaneous regression has limited our understanding of what prevents immunosurveillance from achieving complete elimination, even though, macroscopically, the tumor seems to be rejected. Understanding the mechanisms that regulate immunosurveillance may provide information that could be important in the development of anti-tumor vaccines and the optimization of cancer immunotherapy.

Key to understanding the mechanisms that regulate immunosurveillance is the identification of regulatory cytokines and their sources. It has been shown that T helper cell type 2 (T_H2) cytokines down-regulate anti-tumor immunity[5,6]. It has also been shown that interleukin 4 (IL-4) plays a major role in inducing T_H2 responses[7,8], acting through the IL-4 receptor (IL-4R)[9] and its downstream signal transducer and activator of transcription 6 (STAT6)[10–12]. IL-4R is a heterodimeric complex composed of an IL-4Rα chain and a second receptor chain. The type 1 IL-4R, which consists of IL-4Rα and a common γ chain, binds only IL-4[13–15]. The type 2 IL-4R, which is composed of IL-4Rα and IL-13Rα1 chains, binds not only IL-4, but also IL-13[16]. Because the type 2 IL-4R is shared, IL-13 has similar biological functions to IL-4[17]. However, IL-4 and IL-13 also have diverse functions, such as the ability to induce T cell proliferation[18], which results from the distinct distribution of the two classes of receptors. Although recent studies have demonstrated the importance of IL-13 in the effector limb of T_H2 responses in some infectious diseases[19–22] and allergies[23,24], its role in tumor immunosurveillance is still poorly understood.

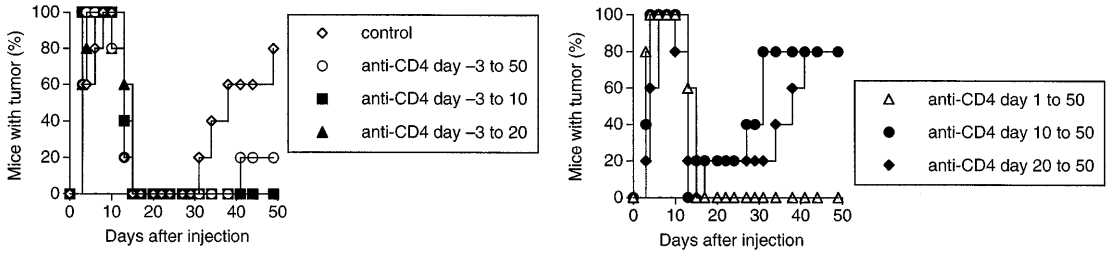

Figure 1. The development of tumors in mice treated with anti-CD4. BALB/c mice received subcutaneous (s.c.) injections of 15-12RM cells to their right flanks. Each group of mice was then inoculated *via* the intraperitoneal (i.p.) route with 0.5 mg of anti-CD4 at the indicated time points. Mice were inoculated with anti-CD4 every day for the first 3 days and then twice a week. (Five mice were used for each group. $P<0.02$ by Log-rank test between control and anti-CD4 from 3 days before treatment to day 10, 20 or 50 after treatment or from day 1 to 50 after treatment. Similar results were obtained in another experiment.)

Natural killer T (NKT) cells, $CD3^+$ T cells that also express NK1.1, are a distinct T cell population including both $CD4^+$ and $CD4^-CD8^-$ cells[25]. They are distributed in various tissues where T cells are found. $CD4^+$ NKT cells can make a large amount of IL-4 promptly after *in vivo* stimulation[26]. NKT cells develop under the restriction of an MHC class I–like molecule, the

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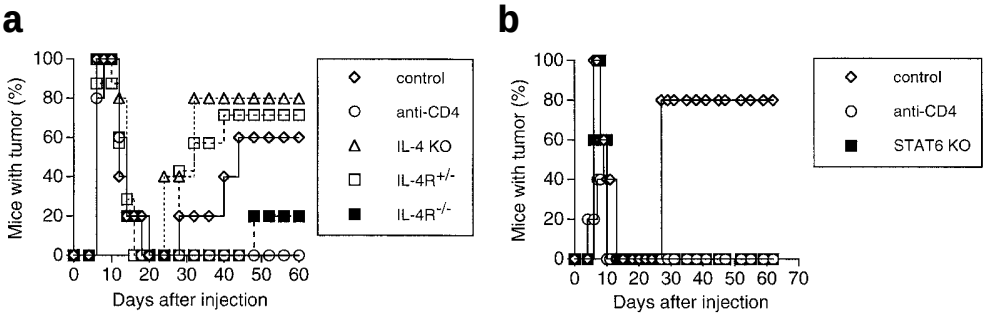

Figure 2. The development of tumors in IL-4–deficient, IL-4R and STAT6-deficient mice. (**a**) Control mice, mice treated with anti-CD4, BALB/c background IL-4 KO, IL-4R KO and heterozygous littermate mice were injected with 1×10^6 15-12RM cells *via* the s.c. route. The mice in the anti-CD4–treated group were treated with anti-CD4 every day from 3 days before 15-12RM injection until the day of the injection and twice a week thereafter. (Five mice were used for each group except for the IL-4R heterozygous group in which seven mice were used. $P<0.05$ by Log-rank test between the control and IL-$4R^{-/-}$ group. The result shown is representative of two experiments with similar results.) (**b**) BALB/c-STAT6 KO mice or wild-type BALB/c mice either untreated or treated with anti-CD4 were injected with 1×10^6 15-12RM cells *via* the s.c. route. Mice in anti-CD4–treated group were treated with anti-CD4 every day from 3 days before 15-12RM injection and twice a week thereafter. (Five mice were used for each group. $P<0.02$ by Log-rank test between the control group and the STAT6 KO group. One representative experiment of three is shown.)

CD1–β_2-microglobulin (β_2M) heterodimer. Mice genetically engineered to be deficient in CD1 have few NKT cells and diminished early production of IL-4[27–29] and IL-13[26].

We have established a mouse tumor model in which the tumor spontaneously regresses after initial growth but then recurs. Using this model, we have shown that regression is mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs) specific for the model tumor antigen HIV gp160[30]. Only tumor cells that lost expression of gp160 mRNA, although they still retained the gene, recurred after spontaneous regression. Interferon γ (IFN-γ) was necessary for optimal elimination of the tumor during the regression phase, and mice depleted of $CD4^+$ T cells before tumor injection were protected from tumor recurrence even though the tumor showed the same initial growth-regression pattern during the early stages of tumor growth. However, because IL-4 knockout (KO, also referred to as deficient) mice were not resistant to tumor regrowth, it was not clear which $CD4^+$ T cell subpopulations negatively regulated the CD8-mediated tumor immunosurveillance and by what mechanism this regulation was accomplished.

We investigated the role of the IL-4R–STAT6 signal pathway and its signaling cytokines, IL-4 and IL-13, in the modulation of immunosurveillance during the spontaneous regression phase of tumor growth. We also determined which $CD4^+$ T cell population is critical for induction of negative regulation of CTL tumor immunosurveillance, and found it was dependent on CD1-restricted NKT cells.

Results

Timing of anti-CD4 treatment

When 15-12RM tumor cells are introduced, by s.c. injection, into the right flank of BALB/c mice at day 0, tumors are detectable within 5 days[30] (**Fig. 1**, control group). The tumors regressed and disappeared after 10–15 days. However, recurrence of the tumors was observed between 20 and 40 days after inoculation and recurrent tumors did not regress (**Fig. 1**, left panel). Tumor recurrence observed in control mice was due to incomplete elimination of tumor cells by $CD8^+$ CTLs during the tumor regression phase. Conversely, in anti-CD4–treated mice, tumor cells were completely eliminated after initial growth and did not recur (**Fig. 1**, left panel)[30]. These results suggested that $CD4^+$ T cells negatively regulated immunosurveillance of tumor cells by CTLs. However, it was not clear at what time point $CD4^+$ T cells began to regulate CTLs or over what time period the regulation was necessary.

To determine when $CD4^+$ T cells regulate CTLs specific for tumor cells, we treated mice with antibody to CD4 over different intervals after 15-12RM injection (**Fig. 1**). All mice in the groups that started to receive antibody to CD4 3 days before 15-12RM injection and stopped receiving antibody at day 10 or day 20 were resistant to tumor recurrence in a similar fashion to the mice treated from day -3 to the end of the experiment. Although delaying the initiation of anti-CD4 treatment by one day did not affect the outcome, treatment started on day 10 was too late to have any impact on the late recurrence (**Fig. 1**, right panel). This suggests that immunosurveillance of CTLs against tumor cells is regulated by $CD4^+$ T cells at an early tumor growth phase. Depletion of $CD4^+$ T cells during the first 10 days was both necessary and sufficient to prevent this regulation.

Down-regulation through IL-4R–STAT6

To clarify whether IL-4 and its signaling pathway are involved in down-regulation of immunosurveillance by CTLs, we used IL-4 and IL-4R knockout mice (**Fig. 2a**). Tumor recurrence occurred in IL-4–deficient mice; indeed, the tumor growth pattern was not significantly different from that of control mice (**Fig. 2a**)[30]. In contrast, in IL-4R–deficient mice, tumors did not recur after the initial growth and regression, although in heterozygous littermates they recurred as in wild-type mice (**Fig. 2a**). Because STAT6 activation is known to be necessary for signaling through the IL-4R signal pathway[10–12], to investigate whether the protection observed in IL-4R, but not in IL-4, knockout mice was dependent on the IL-4R signal pathway, STAT6 knockout mice were inoculated with 15-12RM (**Fig. 2b**). The STAT6-deficient mice were as resistant to tumor recurrence as CD4-depleted mice. This suggested that immunosurveil-

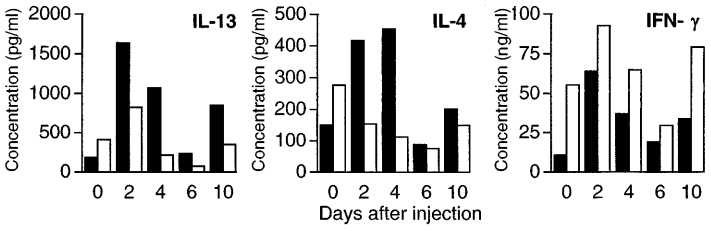

Figure 3. Cytokine production of T cells from 15-12RM–injected BALB/c mice with anti-CD3 stimulation. On days 0, 2, 4, 6 and 10 after injection of 15-12RM cells, two mice per group were killed and their spleen cells collected. A fraction of the cells was used to purify T cells. One hundred thousand freshly isolated T cells from 15-12RM–injected mice were cultured in an anti-CD3–coated plate for 48 h. The culture supernatant was collected and the concentration of each cytokine was determined by ELISA. (Average values for two mice are shown. Similar results were obtained in an additional experiment Control, filled bars; anti-CD4-treated, open bars.)

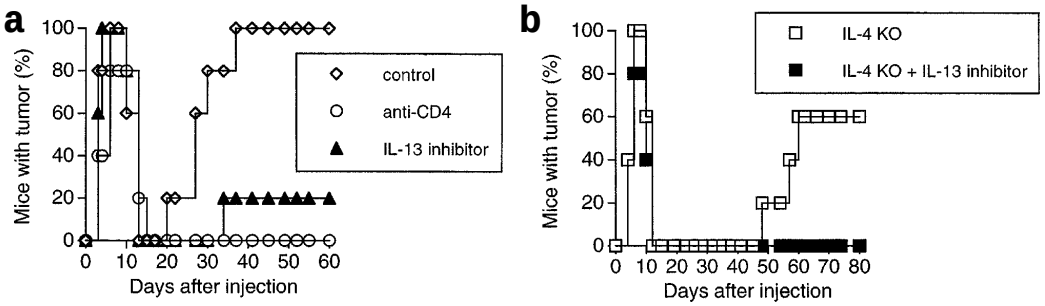

Figure 4. The effect of IL-13 inhibitor on tumor recurrence. (**a**) Control BALB/c mice, mice treated with either anti-CD4 or IL-13 inhibitor or (**b**) BALB/c-IL-4 KO mice either untreated or treated with IL-13 inhibitor were injected *via* the s.c route with 1×10^6 15-12RM cells. Mice treated with anti-CD4 (0.5 mg) were inoculated on days 0, 1, 2, 6 and 10 after 15-12RM injection. Mice in IL-13 inhibitor–treated groups were treated with sIL-13Rα2–Fc (0.2 mg) every other day from day 0 to day 8. (Five mice were used for each group. $P<0.01$ by Log-rank test between the control group and the IL-13 inhibitor group. $P<0.02$ by Log-Rank test between the IL-4 KO group and the IL-4 KO + IL-13 inhibitor group. One representative experiment of two is shown.)

lance by CTLs was limited by the IL-4R–STAT6 signaling pathway, but that this regulation did not require IL-4 itself. Therefore, IL-13, the only other cytokine known to signal through the IL-4R–STAT6 signaling pathway, is likely to play a significant role in this limitation.

Production of cytokines in CD4-depleted mice

Because T cells are major producers of IL-13 and IL-4, we measured *in vitro* production of both cytokines by T cells from 15-12RM–injected mice after anti-CD3 stimulation using enzyme-linked immunosorbent assay (ELISA). Both IL-13 and IL-4 production in T cells from control mice are elevated as early as 2 days after 15-12RM injection (**Fig. 3**). IL-13 and IL-4 production reached a maximum on days 2 and 4, respectively, and then dropped to baseline by day 6 after injection. In T cells from CD4-depleted mice, even though production of IL-13 was up-regulated on day 2, the absolute amount of IL-13 was 50–75% less than that of the cells from 15-12RM–injected control mice. Additionally, IL-4 production was not up-regulated even after 15-12RM injection. Thus, splenic T cells from CD4-depleted mice, which are resistant to tumor recurrence, produced less IL-13 and IL-4.

We also measured production of IFN-γ, which protects against tumor growth in this system[30]. In contrast to IL-13 and IL-4 production, T cells from anti-CD4–treated mice produced 25–100% more IFN-γ than the cells from control mice. These results suggested that removing $CD4^+$ T cells suppresses production of IL-13 and IL-4 but allows more IFN-γ production, which was previously shown to be necessary for the immunosurveillance[30].

IL-13 is critical for the tumor recurrence

These results were compatible with either IL-13 alone being necessary for down-regulation of immunosurveillance or the possibility that either IL-4 or IL-13 was sufficient, and that eliminating both would be necessary to prevent recurrence. To distinguish between these possibilities, we tested the effect of an IL-13 inhibitor (sIL-13Rα2–Fc) in both wild-type and IL-4–deficient mice (**Fig. 4**). The IL-13 inhibitor was inoculated every other day from day 0 to day 8 after 15-12RM injection, but did not prevent the initial growth and regression of the tumor. However, when IL-4 knockout mice, which are not resistant to tumor recurrence, were treated with IL-13 inhibitor they were completely protected from the recurrence. When wild-type mice were treated with the IL-13 inhibitor, the results were similar to those obtained from CD4-depleted mice and IL-13 inhibitor–treated IL-4–deficient mice. These results showed that in our biphasic tumor model, IL-13 is necessary for the tumor recurrence, regardless of the capacity of the mice to make IL-4.

NKT cells can produce IL-13

Treatment during the first 8 days with IL-13 inhibitor protected mice from tumor recurrence (**Fig. 4**). As it has been shown that $CD4^+$ $NK1.1^+$ T cells are among the earliest cells to produce a large amount of IL-4 upon T cell receptor (TCR) activation[31], we examined the ability of $CD4^+$ NKT cells to produce IL-13. As BALB/c mice do not express the *Nk1.1* allele recognized by available monoclonal antibodies, we sorted $CD4^+$ NKT cells from C57BL/6 mice or BALB/c mice congenic for the *Nk1.1* locus. Thus $CD4^+$ T cells were purified, sorted for $CD4^+NK1.1^+$ and

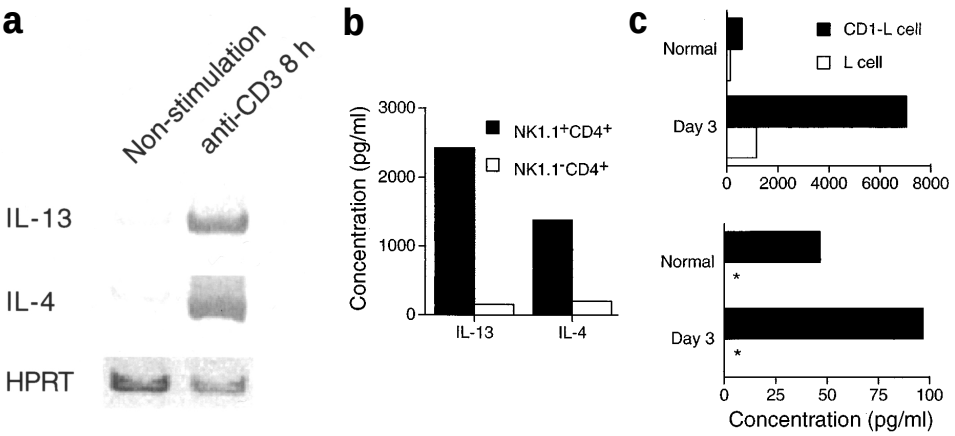

Figure 5. Production of IL-13 by NKT cells. (**a**) Expression of mRNA of IL-13 and IL-4 in $CD4^+$ NKT cells after anti-CD3 stimulation. Freshly sorted splenic $CD4^+$ NKT cells from C57BL/6 mice were stimulated with plate-bound anti-CD3 for 8 h. Cells were collected and mRNA for IL-13 and IL-4 was detected by RT-PCR. (**b**) Production of IL-13 and IL-4 by $CD4^+NK1.1^+$ and $CD4^+NK1.1^-$ T cells. Freshly sorted splenic $CD4^+NK1.1^+$ and $CD4^+NK1.1^-$ T cells from BALB/c mice congenic for the NK1.1 locus were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 h. Supernatants were collected and IL-4 and IL-13 concentrations analyzed by ELISA. (**c**) Production of IL-13 (upper panel) and IL-4 (lower panel) by $CD4^+$ T cells from 15-12RM–injected mice stimulated with CD1-transfected L cells. $CD4^+$ T cells purified from spleen cells from normal and 15-12RM-injected (on day 3 after injection) BALB/c mice were stimulated with CD1-transfected L cells or nontransfected L cells for a week. Anti-CD28 (10 μg/ml) and IL-2 (20 U/ml) were also added in the culture. The culture supernatant was collected and the concentrations of IL-13 and IL-4 determined by ELISA. Asterisks indicate a level under the detection limit (<15.6 pg/ml).

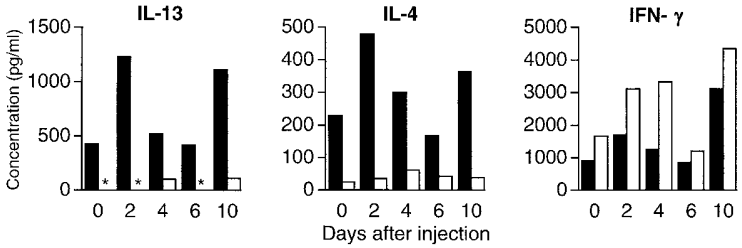

Figure 6. Cytokine production of CD4⁺ T cells from 15-12RM–injected wild-type BALB/c mice and CD1-deficient mice stimulated with anti-CD3. On days 0, 2, 4, 6 and 10 after injection of 15-12RM cells, two mice per group were killed and spleen cells collected. A fraction of the cells was used to purify CD4⁺ T cells. One hundred thousand freshly isolated T cells from 15-12RM–injected mice were cultured in an anti-CD3–coated plate for 48 h. The culture supernatant was collected and the concentration of each cytokines determined by ELISA. (Each value shows the average of two mice. The results are representative of two independent experiments. Asterisks indicate a level under the detection limit, <7.8 pg/ml. Control, filled bars; CD1 deficient, open bars.)

CD4⁺NK1.1⁻ populations and stimulated with anti-CD3. Anti-CD3 stimulation clearly induced IL-13 in CD4⁺NK1.1⁺ T cells, whereas in CD4⁺NK1.1⁻ T cells very little IL-13 was induced (**Fig. 5a,b**). When CD4⁺ T cells from 15-12RM–injected BALB/c mice were stimulated with CD1-transfected L cells (murine fibroblasts), they produced more IL-13 and IL-4 than the cells from nontumor-bearing mice (**Fig. 5c**) but did not produce detectable IFN-γ (data not shown). The lower IL-4 concentrations in **Fig. 5c** compared to **Fig. 5b** may be due to consumption during the 7-day culture in the former, *versus* 48-h in the latter. In contrast, because the purified CD4⁺ T cells do not have IL-13 receptors, that cytokine should not have been consumed. Thus, CD4⁺ NKT cells could indeed make IL-13 and were a primary source of this cytokine. This activity appears to be enhanced in tumor-bearing animals.

CD1-deficient mice and tumor recurrence

If NKT cells are a major source of IL-13, then their absence in CD1-deficient mice should markedly reduce IL-13 production. We examined *in vitro* production of IL-13, IL-4 and IFN-γ in CD4⁺ T cells of 15-12RM–injected CD1-deficient mice, which lack CD1-restricted NKT cells, including CD4⁺ NKT cells, but retain conventional MHC class II–restricted CD4⁺ T cells (**Fig. 6**). CD4⁺ T cells purified from spleen cells of CD1-deficient mice produced only a slight amount of IL-13 and IL-4 after 15-12RM injection, whereas CD4⁺ T cells from wild-type mice up-regulated the production of these cytokines on days 2, 4 and 10 after tumor inoculation. This indicates that the majority of IL-13 production was from, or dependent on, NKT cells. Conversely, the IFN-γ produced was even higher in the cells of CD1-deficient mice than in those of wild-type mice.

Because CD4⁺ NKT cells could produce IL-13, were responsible for much of the IL-13 production and would be depleted by anti-CD4 treatment in addition to conventional CD4⁺ T cells, we hypothesized that NKT cells play a critical role in down-regulation of tumor immunosurveillance by CTLs. To test this hypothesis, CD1-deficient mice were inoculated with 15-12RM (**Fig. 7**). In the CD1-deficient mice, tumors grew initially and regressed in the same time period as in wild-type mice. However, CD1-deficient mice were as resistant to tumor recurrence as anti-CD4–treated wild-type mice. Thus, CD1-restricted NKT cells appeared to be necessary for the down-regulation of immunosurveillance.

In CD4-depleted mice, protection against tumor recurrence appeared to be associated with the higher CTL activity of CD8⁺ T cells immediately *ex vivo*, without restimulation, compared to control mice (**Fig. 8a**)[30]. To determine whether the same applied to CD1-deficient mice, the CTL activity of purified CD8⁺ T cells from 15-12RM–injected CD1-deficient mice was compared with that of the CD8⁺ T cells from tumor-injected control and anti-CD4–treated mice (**Fig. 8**). Freshly isolated CD8⁺ T cells from CD1-deficient (**Fig. 8b**) lysed P18IIIB-pulsed 18Neo cells at a low level without *in vitro* stimulation, whereas CD8⁺ T cells from intact tumor-bearing mice did not kill P18IIIB-pulsed 18Neo cells without restimulation (**Fig. 8c**). Thus, the presence of NKT cells led to reduced CTL activity immediately *ex vivo*.

Taken together, our results suggest that IL-13, produced either by CD1-restricted CD4⁺ NKT cells or possibly by other cells dependent on NKT cells at an early growth phase of the tumor (within 10 days), triggers negative regulation of CTL immunosurveillance and that this negative regulation is mediated through the IL-4Rα–STAT6 pathway.

Discussion

In a mouse tumor model in which tumors spontaneously regress after initial growth, and then recur, CD8⁺ CTLs mediate regression after initial tumor growth. The tumor recurs when CD4⁺ T cells prevent complete eradication of the tumor by CTLs[30]. We determined which cytokine and T cell population down-regulate CTL tumor immunity preventing complete tumor elimination and allowing recurrence after the regression. The down-regulation of CTL immunosurveillance was dependent on IL-4Rα

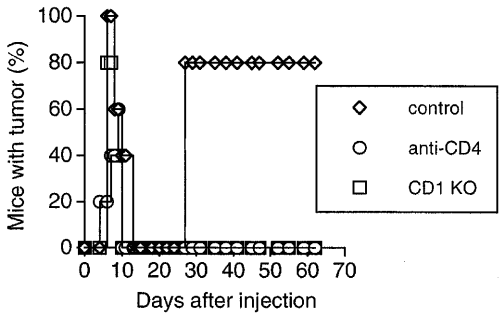

Figure 7. The development of tumors in CD1-deficient mice. BALB/c background CD1 KO mice and wild-type BALB/c mice either untreated or treated with anti-CD4 were injected with 1×10^6 15-12RM cells *via* the s.c. route. Mice in the anti-CD4–treated group received antibody (0.5 mg) starting 3 days before 15-12RM injection and twice a week thereafter. (Five mice were used for each group. $P<0.02$ by Log-rank test between the control group and the CD1 KO group. One representative experiment of four is shown.)

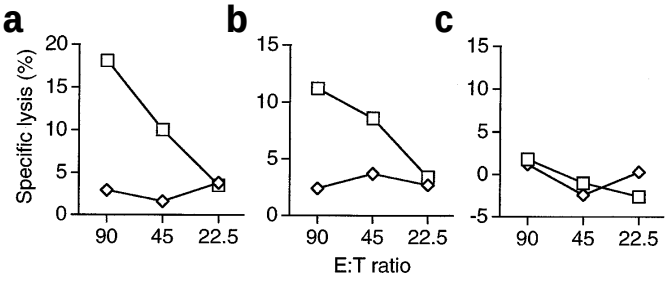

Figure 8. CTL activity of CD1-deficient mice injected with 15-12RM. Freshly isolated CD8⁺ T cells purified from splenocytes of (**a**) anti-CD4–treated (**b**) CD1 KO and (**c**) control mice were examined for CTL activity at 14 days after 15-12RM injection against 18Neo targets pulsed with P18IIIB (1 μM) or not pulsed, without any *in vitro* stimulation. (One representative experiment of three is shown. ◇, targets without peptides; □, targets with peptides.)

signaling and activation of its downstream transcriptional factor STAT6. Using IL-4–deficient mice and sIL-13Rα2–Fc we showed that, of the two cytokines that signal through the IL-4R–STAT6 pathway[16,32], IL-4 was neither sufficient nor necessary for tumor recurrence, whereas IL-13 was necessary. This is consistent with the observation that T cells and $CD4^+$ T cells from mice resistant to recurrence produced less IL-13 by *in vitro* anti-CD3 stimulation than the cells from susceptible mice.

Because both IL-13 and IL-4 bind the type 2 IL-4R (which consists of IL-13Rα and IL-4Rα) and signal through the IL-4R–STAT6 pathway, we did not expect to find that, although the down-regulation of tumor immunosurveillance was dependent on the IL-4R–STAT6 pathway, it was dependent only on IL-13, not IL-4. A similar IL-4R–STAT6 pathway–dependent and IL-13–dependent but IL-4–independent phenomenon was reported in the expulsion of the nematode parasite *Nippostrongylus brasiliensis*[21]. IL-13–dependent up-regulation of soluble IL-13Rα1 expression can occur in T cells without any detection of IL-13Rα1 on their surface[33]. In addition, another IL-13 receptor (IL-13Rα2) was found, the function of which is still unclear[34]. Thus, although several alternative IL-13 signaling pathways exist, our data suggest that the IL-4R–STAT6 pathway is the only one that is relevant to tumor immunosurveillance.

Among $CD4^+$ cells, $CD4^+$ NKT cells are the primary source of IL-13. IL-13 production by CD1-restricted $CD4^+$ NKT cells is increased in tumor-bearing mice and $CD4^+$ NKT cells are among the earliest cells to produce large amounts of cytokines after stimulation[26]. We therefore hypothesized that $CD4^+$ NKT cells might be necessary in inhibiting immunosurveillance. Using CD1-deficient mice lacking CD1-restricted NKT cells, which proved to be as resistant to recurrence as anti-CD4–treated mice, we confirmed this. In addition, when stimulated with anti-CD3, purified splenic $CD4^+$ T cells from tumor-injected CD1-deficient mice produced almost no IL-13 or IL-4 but more IFN-γ than cells from tumor-injected controls. This was consistent with reports that CD1 knockout mice were deficient in early IL-4 production[27–29] and that the β_2M knockout mice were deficient in early IL-4 and IL-13 production *in vivo*[26]. Therefore, much of the IL-13 production was dependent on NKT cells and negative regulation of tumor immunosurveillance by IL-13 was dependent on, and probably triggered by, $CD4^+$ NKT cells.

NKT cells have the potential to produce the T_H1 cytokine, IFN-γ, and T_H2 cytokines, IL-4, IL-5 and IL-10[26,31]. NKT cells can mediate anti-tumor immunity[35,36] (in contrast to inhibiting it, as shown here) by producing a large amount of IFN-γ when stimulated with their specific glycolipid antigen α-galactosylceramide (α-GalCer)[37,38], but α-GalCer has not been found in mammalian cells[39]. On the other hand, when $CD4^+$ NKT cells are stimulated with CD1-transfected L cells *in vitro* they make large amounts of IL-4 rather than IFN-γ [31]. In addition, NKT cells recognize phospholipids such as phosphatidylinositol, which are produced in normal and tumor cells[40]. CD1-restricted $CD4^+$ T cells may exist that recognize antigens from a cellular compartment different from that of antigen for NKT cells that recognize α-GalCer and that also promptly express cytokines after stimulation[41]. $CD4^+$ T cells from tumor-bearing mice up-regulate production of IL-13 and IL-4 when stimulated with CD1-transfected L cells, suggesting that the relevant antigen may not be tumor-specific. In contrast, loss of NKT cells in CD1 knockout mice leads to decreased production of IL-13 as well as increased anti-tumor antigen-specific CTL activity directly *ex vivo* without restimulation *in vitro*. Therefore, $CD4^+$ NKT cells recognizing antigens other than α-GalCer from tumor cells produce IL-13 (or permit other cells to do so) early after tumor injection, thereby down-regulating tumor immunosurveillance. In contrast, $CD4^-CD8^-$ NKT cells, which are not depleted by anti-CD4 treatment, do not appear to play a role.

Although $CD4^+$ NKT cells can produce IL-13 and up-regulate production in tumor-bearing mice, IL-13 cannot bind to T cells. Thus, IL-13 must act on another cell bearing the IL-4Rα–IL-13R receptor and STAT6 pathway, such as macrophages and/or dendritic cells, to reduce the CTL activity that mediates immunosurveillance. IL-13, although necessary, may not be sufficient to mediate the effect. The mechanisms induced by IL-13 remain to be determined. It is unclear whether IL-13 from NKT cells is sufficient to induce downstream events to down-regulate tumor immunosurveillance or whether IL-13 production or action must be amplified though T_H2 cells, which are known to be another source of IL-13[17]. To address this question, the behavior of MHC class II–deficient mice or equivalent mice should be tested. Because BALB/c mice, on which our tumor model is based, have two pairs of MHC class II genes, *I-A* (*H2-Aa*, *H2-Ab*) and *I-E* (*H2-Ea*, *H2-Eb*) that would have to be disrupted, MHC class II–deficient mice on the BALB/c background are not available. Therefore, we could not directly examine the role of MHC class II–restricted $CD4^+$ T cells by depletion of these independent of $CD4^+$ NKT cells. Also, we presume that not all conventional $CD4^+$ helper cells are depleted by *in vivo* treatment with anti-CD4, although in the peripheral blood mononuclear cells (PBMC) compartment we found a 98% reduction in $CD4^+$ T cells (data not shown), as we and others have shown the importance of conventional $CD4^+$ T cells helping CD8-dependent responses, including tumor rejection[42–45]. It is not clear whether the sole role of NKT cells, in this process, is to make IL-13 or whether they contribute through additional mechanisms.

Immunization of mice against the model tumor antigen HIV gp160 protected them from initial growth but could not prevent tumor growth at the later, recurrent phase[30]. Tumor cells from the recurrent stage were not killed by CTLs raised against the original tumor cells, in a manner that was similar to observations of clinical cancer patients. Although vaccination induces tumor regression, the recurrent tumor sometimes lacks the original tumor antigen. Therefore, our finding that neutralization of IL-13 is sufficient to prevent tumor recurrence may provide another approach for improvement of cancer immunotherapy. IL-13 inhibitors may act in synergy with other specific cancer immunotherapies such as cancer vaccines or T cell–adoptive immunotherapy. Similarly, the discovery that NKT cells play a critical role in preventing tumor immunosurveillance may lead to the development of new approaches, by down-regulating these cells, to enhance immunosurveillance.

Methods

Mice. Female BALB/c mice were purchased from Charles River Breeding Laboratories (Frederick, MD). IL-4 knockout mice[46] on a BALB/c background were obtained from the Jackson Laboratory (Bar Harbor, ME). The BALB/c mouse strain congenic for the C57BL/6 *Nk1.1* locus was provided by A. Scalzo and W. Yokayama[47]. C57BL/6 mice, IL-4R knockout[9], STAT6 knockout[12] and CD1 knockout[29] mice with the BALB/c background were bred under pathogen-free conditions. All mice were maintained in a pathogen-free animal facility and were used at 6–10 weeks of age. Animal experiments were all approved by the National Cancer Institute Animal Care and Use Committee.

Tumor cells. 15-12RM tumor cells were made by transfecting BALB/c 3T3 fibroblasts first with HIV-1 IIIB gp160 and then with Ras and Myc to make them tumorigenic[30]. This and control BALB/c 3T3 18Neo cells (transfected with *neo*^{r} only)[48] were maintained in complete T cell medium (CTM) which consisted of RPMI 1640 with 10% fetal calf serum (FCS), L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin and 2-mercaptoethanol (5×10^{-5} M) containing geneticin (200 μg/ml) (Sigma, St. Louis, MO).

Reagents. Purified rat anti-mouse CD4 (GK1.5)[49] was obtained from the Frederick Cancer Research and Development Center, NCI (Frederick, MD). IL-13 inhibitor, a fusion protein of murine IL-13Rα2 and human IgG1 (sIL-13Rα2–Fc), was made as described previously[34] and provided from the Research Support Team at Genetics Institute. Anti-CD3 (2C11)[50], anti-CD28 (37.51), FITC-conjugated anti-CD4 (GK1.5) and PE-conjugated anti-NK1.1 (PK136) were obtained from PharMingen (San Diego, CA). Anti-Ia, anti-CD11b, anti-CD11c, anti-DX5, anti-CD4 and anti-CD8 magnetic beads were from Miltenyi Biotec Inc (Auburn, CA). Recombinant mouse IL-2 was from PharMingen. Trizol, SuperScript cDNA synthesis kit and PCR SuperMixture were from Life Technologies (Rockville, MD).

Tumor inoculation. One million 15-12RM cells in PBS (200 µl) were injected subcutaneously into the right flank of the mouse subjects. To deplete $CD4^+$ cells *in vivo*, mice were inoculated intraperitoneally with 0.2 ml of PBS containing 0.5 mg of anti-CD4 or control rat IgG (Sigma, St. Louis, MO). In some experiments, mice were treated every other day for 8 days with 0.2 mg of sIL-13Rα2–Fc by i.p. injection in PBS (0.1 ml).

***In vitro* activation of T cells, $CD4^+$ T cells and $CD4^+$ NKT cells.** Ninety-six well plates were coated with anti-CD3 (10 µg/ml) overnight at 4 °C. Before use, the plates were washed three times with PBS. Single-cell suspensions of splenocytes from 15-12RM–injected mice were prepared at different time points after the injection. T cells were negatively selected from splenocytes by using magnetic beads coated with antibodies to Ia, CD11b, CD11c and DX5. $CD4^+$ T cells were obtained by depleting $CD8^+$ cells from T cells by using anti-CD8 magnetic beads. The cells were cultured at a density of 1×10^5/well of a 96-well plate in CTM (200 µl). Two days after the stimulation, 100 µl of culture medium was collected from each well and stored at -70 °C until cytokine measurement. In some experiments, $CD4^+$ T cells (1×10^6/well) were stimulated with CD1-transfected L cells[31] or L cells (2×10^4/well) with anti-CD28 (10 µg/ml) and IL-2 (20 units/ml). After a week, culture medium was collected and stored at -70 °C until cytokine measurement.

Spleen cells of C57BL/6 mice or BALB/c mice congenic for the NK1.1 locus were passed through a CD4-enrichment column (Cedar Lane, Westbury, NY) and then sorted for $CD4^+$ $NK1.1^+$ or $CD4^+$ $NK1.1^-$ T cells by using FACStar (Becton Dickenson, Mountain View, CA). To detect cytokine mRNA, the cells (1×10^5/well) were cultured in CTM (200 µl) in a 96-well plate. Eight hours after stimulation cells were collected from each well and stored at -70 °C until RNA extraction. For cytokine measurment, 2×10^4 purified cells were stimulated in 96-well plates coated with monoclonal antibodies to CD3 and CD28 (10 µg/ml each). Supernatants were collected at 48 h and analyzed for IL-4, IL-13 and IFN-γ.

Cytokine assays. The concentrations of IL-4, IL-13 (R&D, Minneapolis, MA or Endogen, Woburn, MA) and IFN-γ (R&D) in the culture supernatant were determined by ELISA kit according to the manufacturer's instructions. All samples were analyzed in triplicate.

CTL assay. Cytotoxic activity of $CD8^+$ T cells against several target cells was measured by a 4-h ^{51}Cr-release assay. $CD8^+$ T cells were purified from splenocytes by depletion of B cells, macrophages, DC, NK cells and $CD4^+$ cells using magnetic beads (Miltenyi Biotec Inc., Auburn, CA). The percentage of specific ^{51}Cr release was calculated as follows:

$$\frac{\text{(experimental release-spontaneous release)}}{\text{(maximum release-spontaneous release)}} \times 100$$

Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated without added effector cells.

Detection of cytokine mRNA by RT-PCR. Total RNA was extracted from sorted $CD4^+$ NKT cells with Trizol reagent. Using the Suprescript cDNA synthesis kit cDNA was synthesized according to manufacturer's instructions. PCR was done with Supermixture containing primers (0.2 µM) by the GeneAmp 9700 PCR system (Perkin-Elmer, Norwalk, CT). The PCR was run for 28 cycles of 94 °C for 30 s, 55 °C for 1 min, 7 °C for 1 min. The primers for IL-4 were from Promega (Madison, WI). The sequence of the primers used were follows: IL-13 (sense) 5′–GACCCAGAGGATATTGCATG–3′; IL-13 (antisense) 5′–CCAGCAAAGTCTGATGTGAG–3′; HPRT (sense) 5′–GTTGGATACAGGCCAGACTTTGTTG–3′; HPRT (antisense) 5′–TCGGTATCCGGTCGGATGGGAG–3′.

Statistical analysis. The data were analyzed for statistical significance using a Log-Rank test. The data were considered significant at $P<0.05$. The Log-rank test was performed with the software JMP version 3.1.5, from Institute Inc. (Cary, NC.)

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