Brief Definitive Report

Immunotherapy of Cytotoxic T Cell–resistant Tumors by T Helper 2 Cells: An Eotaxin and STAT6-dependent Process

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Abstract

Currently most attempts at cancer immunotherapy involve the generation of $CD8^+$ cytotoxic T lymphocytes (CTLs) against tumor-associated antigens. Many tumors, however, have been immunoselected to evade recognition by CTLs and thus alternative approaches to cancer immunotherapy are urgently needed. Here we demonstrate that $CD4^+$ T cells that recognize a secreted tumor-specific antigen and exhibit a cytokine secretion profile characteristic of Th2 cells, are capable of clearing established lung and visceral metastases of a CTL-resistant melanoma. Clearance of lung metastases by the Th2 cells was found to be totally dependent on the eosinophil chemokine, eotaxin, and partially dependent on the transcription activator signal transducer and activator of transcription 6 (STAT6), with degranulating eosinophils within the tumors inducing tumor regression. In contrast, tumor-specific $CD4^+$ Th1 cells, that recruited macrophages into the tumors, had no effect on tumor growth. This work provides the basis for a new approach to adoptive T cell immunotherapy of cancer.

Key words: Th2 cells • tumor immunotherapy • immune evasion • eosinophils • eotaxin

Introduction

There is ample evidence that many tumors express antigens that can be recognized by the adaptive immune system (1) and potentially can be used to induce an antitumor immune response. Until now most cancer immunotherapy studies have focused on the generation of $CD8^+$ CTLs that recognize tumor antigens, in association with MHC class I molecules, on tumor cells. With mounting evidence that many tumors have been immunoselected to evade recognition by CTLs (2) alternative approaches to cancer immunotherapy need to be investigated. In this context, the induction of tumor-specific $CD4^+$ T cells has been largely ignored, except when optimum activation and development of $CD8^+$ CTLs is thought to depend on help from $CD4^+$ T cells (3, 4).

Based on their profile of secreted cytokines, $CD4^+$ T cells have been frequently subdivided into two subpopulations, Th1 and Th2, with the Th1 cells predominantly producing IL-2 and IFN-γ and the Th2 cells preferentially secreting IL-4, IL-5, and IL-10 (5). It is generally thought, however, that only Th1 cells provide help to $CD8^+$ CTLs, the Th2 cells regulating humoral immune responses (5). On the other hand, there are a number of reports suggesting that both $CD4^+$ Th1 and Th2 cells may act independently of $CD8^+$ CTLs and play a direct role in the elimination of tumors (6–8). In these cases tumor eradication may be mediated by tumoricidal myeloid cells recruited into the tumors (6, 9, 10) or by anti-angiogenic cytokines, such as IL-4, secreted by $CD4^+$ T cells (11).

To elucidate the direct antitumor activity of Th1 and Th2 cells, particularly against tumors resistant to CTL lysis, we exploited a highly metastatic and CTL-resistant tumor cell line (B16 mouse melanoma). This line was transfected with the chicken protein, OVA, to yield the B16-OVA melanoma line, the OVA acting as a surrogate secreted tumor-specific antigen. Polyclonal populations of OVA-specific $CD4^+$ T cells, that were polarized to produce either Th1 or Th2 cytokines, were then examined for their ability to eliminate established metastases of the B16-OVA melanoma. Here we report that OVA-specific Th2 cells, but not Th1 cells, are capable of clearing metastases produced by the B16-OVA melanoma, with tumor clearance being dependent on the eosinophil chemokine,

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eotaxin, the IL-4 receptor/signal transducer and activator of transcription 6 (STAT6) signaling pathway and degranulating eosinophils within the tumors.

Materials and Methods

Mice and Tumor Cell Lines. C57BL/6 mice and various gene knockout mice on a C57BL/6 background were used between 6–8 wk of age. All animal experimental protocols were approved by the Australian National University Animal Experimentation Ethics Committee. The B16-F1 melanoma cell line was transfected with OVA as reported previously (12), to yield the B16-OVA line.

OVA-specific T Cell Populations. OVA specific $CD4^+$ Th1, Thi and Th2 cells were generated as described previously (13). Briefly, C57BL6 mice were primed with OVA in alum and 6 d later the splenocytes from the immunized mice cultured for 4 d with OVA alone (Thi cells) or in combination with IL-12 and a neutralizing anti–IL-4 mAb to generate Th1 cells or IL-4 and a neutralizing anti–IFN-γ mAb to generate Th2 cells. $CD4^+$ T cells, purified by magnetic beads, were then transferred into tumor bearing mice or restimulated in vitro with OVA to confirm the cytokine profile of the $CD4^+$ T cells.

Tumor Metastasis Assays. B16 and B16-OVA melanoma cells (3×10^5) were injected intravenously into recipient mice. After 7 d, when lung metastases were macroscopically visible (typically >150 metastases/mouse), Th1, Thi, or Th2 cells (2×10^7) were transferred intravenously into the tumor-bearing mice. 14 d after tumor challenge, mice received a second intravenous injection of T cells (2×10^7). The antitumor activity mediated by the transferred cells was determined by counting the number of lung metastases 18–20 d after tumor challenge. Tumor-bearing lungs were fixed in 10% phosphate-buffered formalin 72 h after the first T cell transfer, sectioned, and stained with Carbol's chromotrope-hematoxylin for identification of eosinophils. Fixed lung sections were also immunostained either with an anti-major basic protein (MBP) antibody (14) to identify eosinophils or with the F4/80 mAb to identify intratumoral macrophages.

Cytotoxicity Assays. Eosinophil cytotoxicity was determined by a 6-h ^{51}Cr-release assay using B16-OVA melanoma cells as target cells. Eosinophils were isolated from the peritoneal cavity of naive IL-5 transgenic C57Bl/6 mice by sorting, based on forward and side light-scatter, using a Becton Dickinson FACStar$^{Plus™}$ flow cytometer with purity of the sorted eosinophils being ≥98%. Eosinophils were lysed by freeze-thawing three times and the eosinophil lysates then incubated with B16-OVA melanoma targets.

Results

$CD4^+$ Th2 Cells, but Not Th1 Cells, Are Able to Eradicate Established Melanoma Metastases. Initial studies demonstrated, by ELISA, that the B16-OVA melanoma secreted low levels of OVA, confluent cultures only containing ~100 ng/ml in the culture medium. The B16-OVA cells also exhibited comparable metastatic activity to the B16 parent line and were largely resistant to lysis by OVA-specific $CD8^+$ CTLs both in vitro and in vivo, except when very high avidity CTLs were used (M. Estcourt and I. Ramshaw, personal communication). Serological studies also revealed that the B16-OVA cells expressed no detectable class I or class II MHC antigens on their surface (unpublished data). Polyclonal populations of OVA-specific

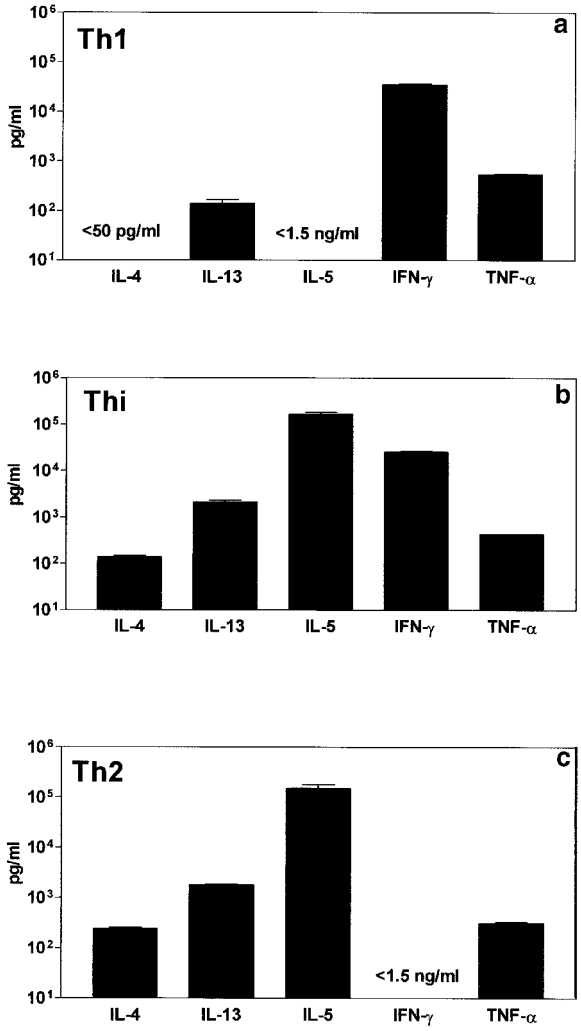


Figure 1. Cytokine production by different polyclonal populations of OVA-specific $CD4^+$ T cells. (a) Th1, (b) Thi, and (c) Th2 populations of T cells. Cytokine production (pg/ml) was measured by ELISA in culture supernatants following OVA restimulation of the different T cell populations for 48 h. Error bars represent SEM.

$CD4^+$ Th1 and Th2 cells were then prepared by culturing splenocytes from OVA immunized mice with OVA in the presence of either IL-12 and anti–IL-4 (to generate OVA-specific Th1 cells) or IL-4 and anti–IFN-γ (to generate OVA-specific Th2 cells; reference 13). Culturing the splenocytes in OVA alone resulted in an intermediate Th1/Th2 phenotype (termed OVA-specific Thi cells). To confirm the Th phenotype of the different T cell populations, the cytokine profile of the different $CD4^+$ T populations after OVA restimulation in vitro was assessed. As expected, the OVA-specific Th1 cells produced no detectable IL-4 and IL-5 but high levels of IFN-γ (Fig. 1 a) whereas the OVA-specific Th2 population secreted IL-4 and IL-5 but no detectable IFN-γ (Fig. 1 c). By contrast, the Thi population produced both Th1 and Th2 cytokines (Fig. 1 b). IL-13 and TNFα were secreted by all three OVA-specific T cell populations (Fig. 1, a–c), although the Th1 cells released substantially less IL-13 than the Thi and Th2 populations.

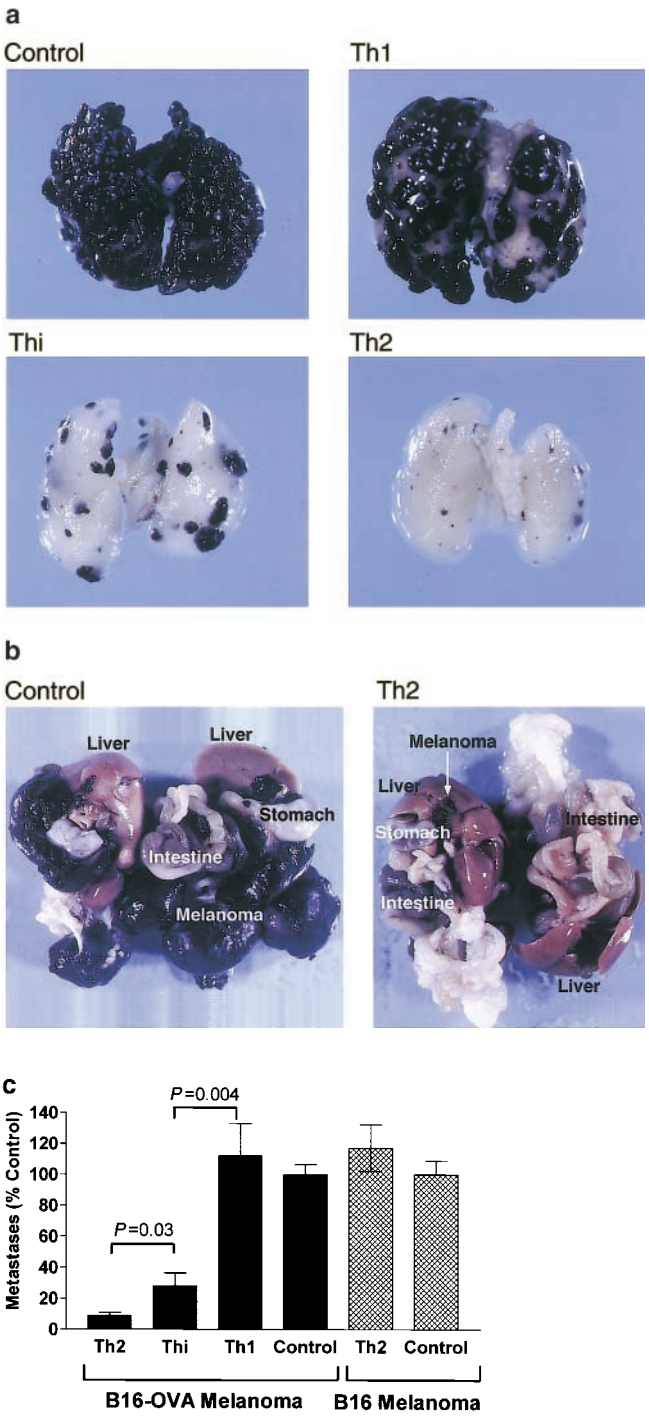
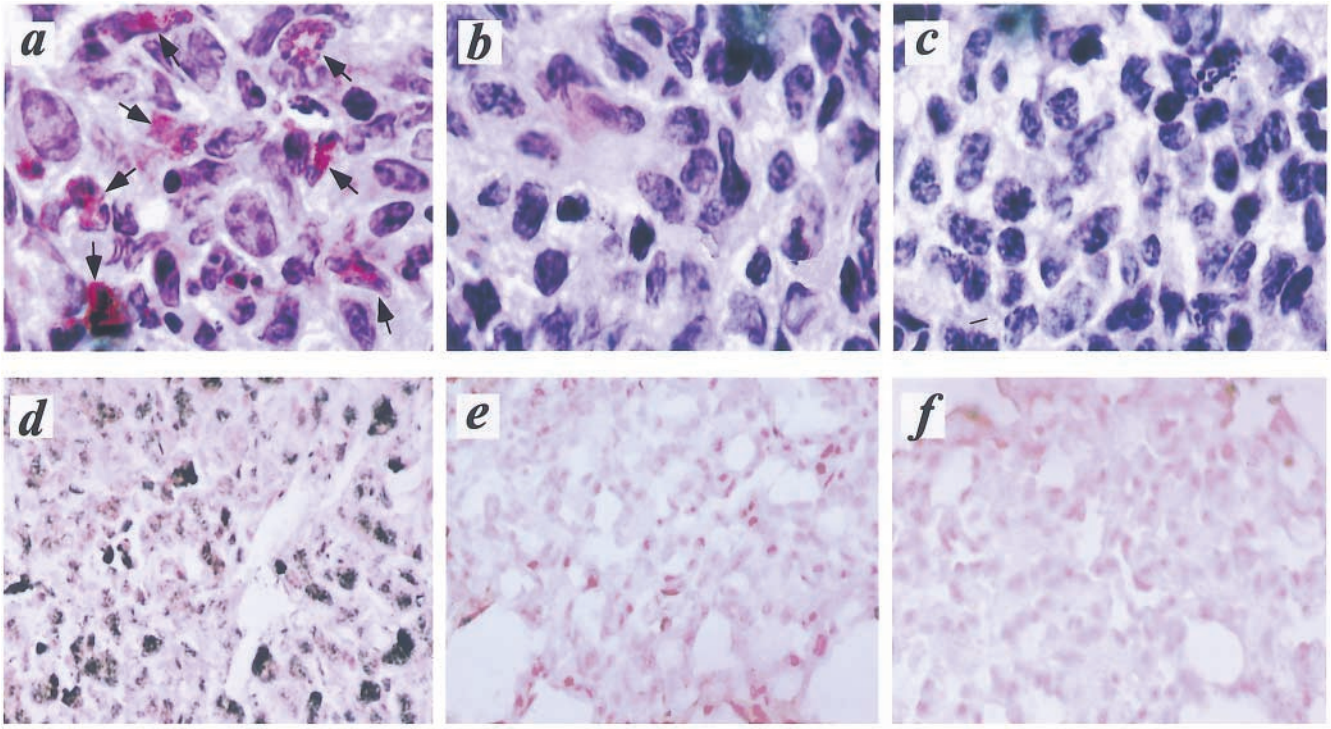


Figure 2. Adoptively transferred populations of OVA-specific $CD4^+$ Thi and Th2 cells, but not Th1 cells, inhibit the growth of lung metastases produced by an OVA-transfected B16 melanoma (B16-OVA). (a) Macroscopic view of the lungs from an untreated (Control) mouse or mice receiving OVA-specific Th1, Thi, or Th2 cells. (b) Visceral melanoma metastases in the organs of an untreated (Control) mouse and a mouse receiving OVA-specific Th2 cells. (c) Quantification of clearance of melanoma lung metastases, relative to the untreated control, by the different OVA-specific $CD4^+$ T cell populations. Untreated control mice usually contained 100–200 melanoma metastases in their lungs. Error bars represent SEM (n = 6–7 mice).

To test the antitumor activity of the Th1 and Th2 cells, syngeneic C57BL/6 mice were injected intravenously with 3×10^5 B16-OVA tumor cells and 7 d later, when >100 tumor metastases were macroscopically visible in the lungs of each mouse, 2×10^7 OVA-specific $CD4^+$ T cells were adoptively transferred into each tumor bearing animal. A second dose of OVA-specific $CD4^+$ T cells was administered 14 d after tumor cell injection and lung metastases quantified 18–20 d after tumor challenge. Compared with untreated control animals, the OVA-specific Th1 cells had no effect on tumor growth (Fig. 2, a and c). By contrast, both the OVA-specific Thi and Th2 populations significantly reduced the number of surface lung metastases, with the Th2 cells reducing metastases by >90%. Furthermore, the lung metastases that persisted in the Th2 cell–treated animals were much smaller than in the other treatment groups (Fig. 2 a). Indeed, histological examination of lungs from Th2 cell recipients revealed that micro-metastases were only detectable immediately beneath the lung surface, with the remainder of the lung tissue being totally devoid of tumor cells. A single dose of OVA-specific Th2 cells reduced the number of surface lung metastases by ~70–80% whereas a second dose of Th2 cells resulted in >90% reduction, thus in all subsequent experiments two doses of $CD4^+$ T cells were administered.

After multiple passages in vitro the B16-OVA melanoma line gained the ability to metastasize to most abdominal organs (i.e., liver, stomach, duodenum, small intestine, colon, spleen) after intravenous injection. When OVA-specific Th2 cells were transferred to mice receiving multiply passaged B16-OVA cells, not only clearance of tumors from the lungs resulted but a dramatic inhibition of tumor growth in abdominal organs occurred (Fig. 2 b), indicating that the antitumor effect of the OVA-specific Th2 cells is not restricted to the pulmonary compartment. In addition, the antitumor activity of the Th2 cells was OVA-specific, as the OVA-specific Th2 cells had no effect on the lung metastases of the parent B16 melanoma cell line (Fig. 2 c).

Eradication of Melanoma Metastases by $CD4^+$ Th2 Cells Is Associated with an Influx of Eosinophils into the Tumors. Histological examination of B16-OVA lung tumors 72 h after the first injection of the different $CD4^+$ T cell populations revealed that there were considerable numbers of eosinophils in the tumors of mice receiving OVA-specific Th2 (Fig. 3 a) and Thi cells, i.e., approximately a 30-fold increase above the background eosinophil content of tumors in mice receiving no $CD4^+$ T cells (Fig. 3, c and g). By contrast, in mice receiving OVA-specific Th1 cells only background levels of eosinophils were detected in the lung tumors (Fig. 3, b and g). The influx of eosinophils into tumors was OVA-specific as the Th2 cells were unable to recruit eosinophils into the OVA-deficient tumors produced by the parent B16 melanoma (1.9 ± 0.3 eosinophils/high power field (HPF) were detected in the B16 tumors of Th2 cell recipients compared with 1.3 ± 0.2 eosinophils/HPF in the B16 tumors of control mice). Also, when OVA-specific Th2 cells were labeled with the intracellular fluorescent dye CFSE (15) they were found, by immunofluorescence microscopy, to have localized in the B16-OVA lung tumors (unpublished data).



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	Th2	Th1	Control
Eos	31.2 ± 5.1	1.2 ± 0.4	1.3 ± 0.2
Mac	3.1 ± 0.4	7.9 ± 0.9	1.9 ± 0.2

Figure 3. Clearance of B16-OVA lung metastases by OVA-specific $CD4^+$ T cells correlates with the influx of eosinophils, but not macrophages, into tumors. Eosinophil staining (a–c) and immunohistochemical detection of eosinophil MBP (d–f) in sections of lung tumors (×400) from a control mouse or from mice 72 h after receiving OVA-specific Th2 or Th1 cells. (g) Number of either eosinophils (Eos), detected by staining with Carbol's chromotrope-hematoxylin, or macrophages (Mac), detected with the F4/80 mAb/tumor HPF (± SEM) in untreated (control) mice or mice receiving Th2 or Th1 cells.

The presence of eosinophils in the tumors of mice receiving OVA-specific Th2 cells, but not in the tumors of either control or Th1 cell–treated animals, was confirmed immunohistochemically by staining for the eosinophil granule protein, MBP (Fig. 3, d–f). In the case of macrophages the converse was true, as we observed an influx of macrophages approximately fourfold above background in the tumors of mice receiving OVA-specific Th1 cells ($P = 0.0004$ compared with controls), with macrophage numbers barely increasing above background levels in tumors from Th2-treated animals (Fig. 3 g). Thus, Th1 tumor-specific immunity, in contrast to Th2 immunity, had no effect on tumor growth, despite considerable numbers of macrophages becoming localized in the tumors.

Elimination of Melanoma Metastases by $CD4^+$ Th2 Cells Is Dependent on Eotaxin and STAT6. A number of gene knockout mice were used to further probe the molecular and cellular basis of the inhibition of tumor growth by Th2 cells. OVA-specific Th2 cells were capable of clearing B16-OVA tumor metastases in $RAG1^{-/-}$ mice, the Th2 cells being almost as effective as in wild-type C57BL/6 mice (Fig. 4 a). Eosinophil influx into the tumors was also unchanged in $RAG1^{-/-}$ mice (Fig. 4 b). As $RAG1^{-/-}$ mice lack lymphocytes these findings substantiate that Th2 cell–mediated antitumor immunity occurs independent of recipient T cells, NKT cells, and B cells. In contrast, the Th2 cells were substantially less effective at reducing lung metastases in STAT6–deficient ($STAT6^{-/-}$) mice than in wild-type recipients ($P = 0.001$), although there was still significant inhibition of tumor growth in the STAT6-deficient mice ($P=0.02$; Fig. 4 a). STAT6 plays an essential role in signaling via the IL-4 receptor (IL-4R; reference 16), with the IL-4R–STAT6 pathway inducing secretion of the eosinophil chemokine, eotaxin, by epithelial cells (17). Interestingly, STAT6 deficiency also resulted in only a partial reduction in the eosinophil content of tumors in the Th2 recipient mice (Fig. 4 b), indicative of a STAT6-independent pathway of eosinophil recruitment. Such a pathway has been described for eotaxin induction in human epithelial cells (18). Conversely, IL-5, a cytokine known to regulate eosinophil expansion and recruitment from the bone marrow into the circulation (19), played no

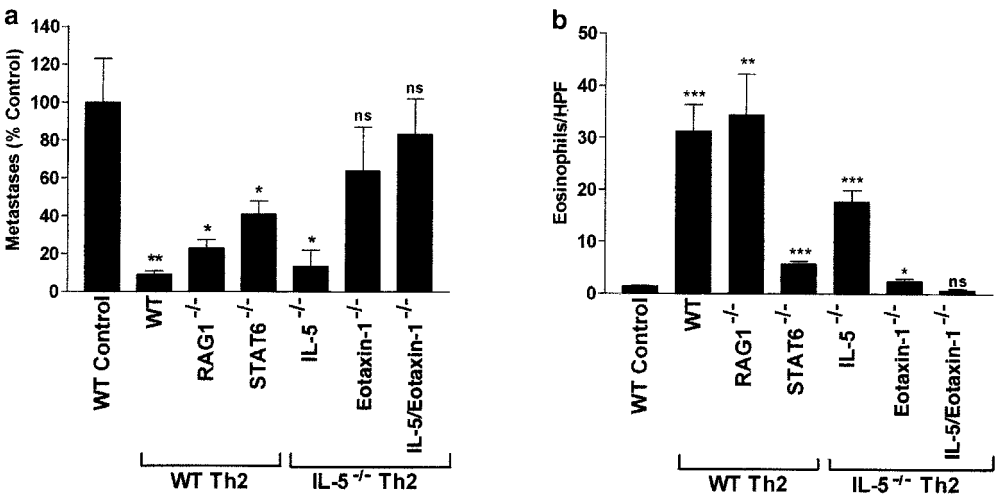

Figure 4. Clearance of B16-OVA lung metastases and recruitment of eosinophils into tumors by OVA-specific Th2 cells is independent of recipient lymphocytes and IL-5 but is STAT6 and eotaxin dependent. Ability of OVA-specific Th2 cells, either WT or IL-5 deficient, (a) to inhibit lung tumor metastases or (b) to induce an eosinophil influx into the lung tumors of various gene knockout mice. Asterisks refer to significant difference between treatments and WT controls with $^{*}P < 0.05 > 0.01$, $^{**}P < 0.01 > 0.001$, $^{***}P < 0.001 > 0.0001$, and ns, not significant.

role in tumor clearance as IL-5–deficient Th2 cells, when transferred to IL-$5^{-/-}$ recipients, were unimpaired in their ability to eliminate lung metastases or recruit eosinophils into the tumors (Fig. 4, a and b). As IL-5 is produced by Th2 cells (Fig. 1 c), Th2 cells generated in IL-$5^{-/-}$ mice were used in these experiments. The critical role played by eotaxin in Th2-mediated tumor regression was evident in eotaxin-deficient mice which failed to clear tumor metastases after the administration of OVA-specific IL-$5^{-/-}$ Th2 cells (Fig. 4 a). As would be expected, eotaxin deficiency was also associated with low level entry of eosinophils into the tumors (Fig. 4 b). As with the eotaxin-deficient recipients, IL-$5^{-/-}$ Th2 cells were also unable to eliminate B16-OVA tumors in mice deficient in both IL-5 and eotaxin, with eosinophil entry into the tumors being at background levels (Fig. 4, a and b). Previous studies have shown that Th2 cytokines can induce eotaxin production by lung epithelial cells (17, 18). As little or no eosinophil recruitment was observed in tumors in eotaxin-deficient recipients, this indicates that the tumor cells themselves are not eotaxin producers.

Detection of Degranulating Eosinophils in Regressing Tumors. Immunohistochemical studies provided evidence for degranulating eosinophils in the tumors of mice receiving OVA-specific Th2 cells, with eosinophil MBP being detected free of eosinophils in the tumors (Fig. 5 a). Thus it appears likely that cellular toxins, such as MBP, released from eosinophil granules may mediate tumor destruction. When eosinophils were incubated with tumor cells in vitro no lysis of the tumor cells was observed, although eosinophil lysates were found to be cytotoxic (Fig. 5 b), supporting the view that eosinophil granule proteins are tumoricidal. Addition of cytokine containing supernatants from OVA stimulated Th1, Thi, or Th2 cells (Fig. 1), with or without eotaxin, to the eosinophil–tumor cell mixture also resulted in no detectable lysis of the tumor cells (unpublished data). Thus, it appears that in vivo factors provided by the tumor microenvironment facilitate eosinophil degranulation and destruction of the tumor cells.

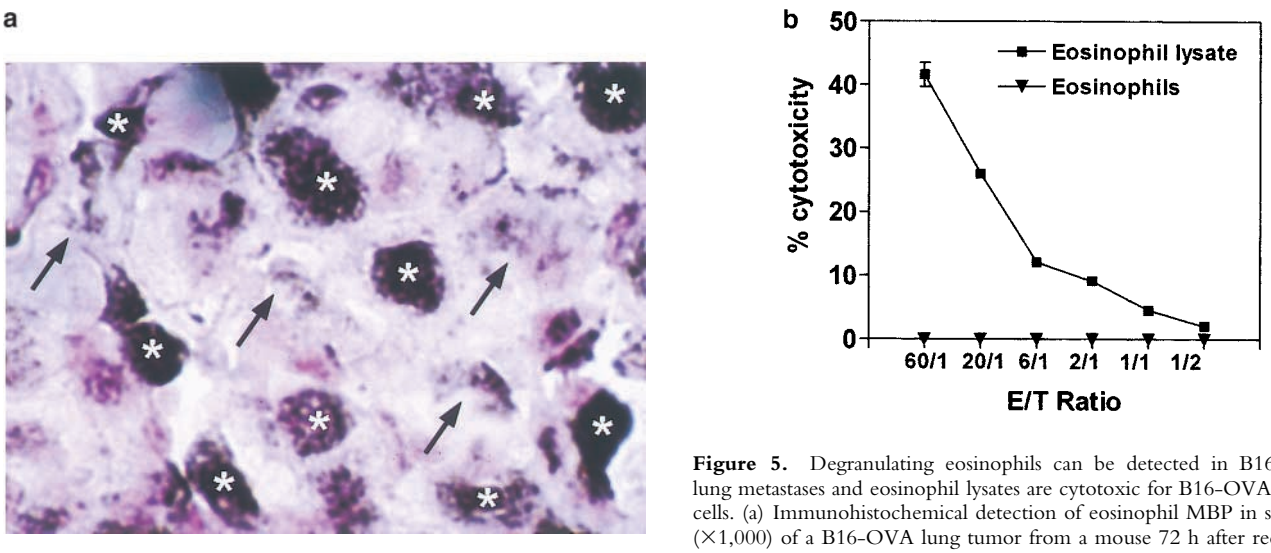

Figure 5. Degranulating eosinophils can be detected in B16-OVA lung metastases and eosinophil lysates are cytotoxic for B16-OVA tumor cells. (a) Immunohistochemical detection of eosinophil MBP in sections (×1,000) of a B16-OVA lung tumor from a mouse 72 h after receiving OVA-specific Th2 cells. Arrows indicate the presence of MBP staining material free of eosinophils, indicative of eosinophil degranulation, with intact eosinophils staining for MBP being highlighted with white asterisks. Staining representative of >10 tumor sections. (b) Ability of eosinophils or eosinophil lysates, at different effector to target cell ratios, to lyse ^{51}Cr-labeled B16-OVA tumor cells.

Discussion

This study indicates that tumor-specific $CD4^+$ T cells with a cytokine profile characteristic of Th2 cells can very effectively eliminate lung and visceral tumor metastases that are resistant to eradication by tumor-specific CTLs. In fact, the study implies that tumor-specific Th2 cells are much more effective against highly metastatic solid tumors than is generally realized. Also, based on histological analyses and the use of a range of gene knockout mice, compelling evidence was obtained that tumor elimination by Th2 cells is dependent upon an influx of eosinophils into the tumors. Of critical importance here was the failure of the Th2 cells to clear lung metastases in eotaxin-deficient recipient mice, the chemokine eotaxin being reported previously to mediate the recruitment of eosinophils into tissues (20). Furthermore, the reduced capacity of Th2 cells to eliminate tumors in STAT6-deficient mice is consistent with eotaxin playing an essential role in tumor clearance as eotaxin production by lung epithelial cells is largely dependent on signaling by the IL-4R–STAT6 pathway (21, 22). In contrast, recipient $CD8^+$ CTLs, $CD4^+$ T cells, NKT cells, and B lymphocytes are unlikely to participate in tumor elimination as the Th2 cells were active in RAG1-deficient recipients. Based on our data it appears likely that tumor cell eradication induced by Th2 cells is mediated by cytotoxic proteins, such as MBP, released by degranulating eosinophils, although we were unable to identify in vitro the factors that induce eosinophil degranulation. It is conceivable, however, that the eosinophils may also be tumoricidal via other mechanisms such as superoxide and nitric oxide production (6).

An intriguing feature of this study is that tumor-specific Th1 cells were unable to inhibit tumor growth despite recruiting macrophages into the tumors. This apparent paradox may be explained by the observation that, rather than being tumoricidal, tumor-infiltrating macrophages are often pro-angiogenic and favor tumor growth (23, 24). In fact, tumor-infiltrating macrophages are associated with a poor prognosis in melanoma and breast cancer patients (24, 25). Nevertheless, there are reports that in certain situations $CD4^+$ Th1 cells can directly eradicate tumors (6, 7). The reason for this discrepancy with our results is probably related to the tumor models employed. For example, in one case $CD4^+$ T cells were examined for their ability to eliminate tumor cells expressing MHC class II molecules (7), whereas in another instance the tumor-specific $CD4^+$ Th1 cells were induced by irradiated, GM-CSF–expressing, tumor cells (6).

There are a number of earlier reports suggesting that eosinophils may be capable of eliminating tumors, with the current study providing definitive evidence that eosinophils are involved in tumor clearance. Tumor cells transfected with IL-4 grow poorly in recipient animals, the poor tumor growth appearing to be associated with an IL-4–induced influx of eosinophils (9, 10), although recent studies suggest that IL-4 can also be anti-angiogenic (11). Similarly, the direct antitumor activity of $CD4^+$ T cells appears to correlate with tumors being infiltrated with eosinophils (6).

A common feature of many spontaneously occurring tumors is that they evade CTL elimination by loss of MHC molecule expression (2), as is the case for the B16 melanoma used in this study. By contrast, as Th2 cells can respond to secreted tumor antigens presented, in association with MHC class II molecules, by bystander antigen-presenting cells, and recruit eosinophils into the tumor that are nonspecifically tumoricidal via soluble factors, this type of immune attack may be less susceptible to immune evasion. Certainly, unlike CTLs, this form of tumor immunity would still be effective against single cells arising within a tumor that lack MHC molecules or the antigen against which the antitumor immune response is directed, the cytotoxic proteins released by degranulating eosinophils killing such cells as "innocent" bystanders. By focusing the immune response on tumor antigens that are essential for cell survival (26), the chances of immune escape would be reduced even further.

Thus, it can be proposed that the induction of Th2 immunity to secreted tumor-specific antigens, and resultant eosinophil-induced tumor regression, represents a viable approach to cancer immunotherapy. Perhaps the success of such an approach is to be expected, as degranulating eosinophils in the lungs of chronic asthmatics are known to cause extensive tissue destruction (27). On the other hand, we observed that Th2 cells rapidly eliminated lung metastases with there being no histological evidence of lung damage or persistent inflammation after tumor clearance. This finding raises the intriguing possibility that tumor-specific Th2 immunity could be continually eradicating micro-metastases with there being no lasting histological changes to indicate that this has actually occurred.

This paper is dedicated to one of the coauthors, Wei Xie, who tragically died of hepatocellular carcinoma while the study was in progress. We thank S. Gruninger, K. Jakobsen, P. Jian, A. Prins, and A. Siqueira for assistance and K. Matthaei for supplying the IL-$5^{-/-}$ and IL-$5^{-/-}$, eotaxin$^{-/-}$ mice. We also thank J. Lee and N. Lee, Mayo Clinic, Scottsdale, AZ, for their kind gift of the MBP mAb.

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