Characterizing the Protective Component of the αβ T Cell Response to Transplantable Squamous Cell Carcinoma

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There is increasing promise that cellular immune response may be manipulated to combat cancer; however, it is also clear that the immune response to cutaneous malignancy comprises different T cell activities that variably inhibit or promote tumor development. Thus, a better understanding of each of these activities is crucial to more effective clinical manipulation. To better characterize the protective anti-tumor effects of αβ T cells, we examined the growth of the transplantable squamous cell carcinoma (SCC) line, PDV, which is markedly inhibited in immunocompetent *versus* αβ T cell-deficient mice. We show that the protective response is composed of $CD8^+$ and interferon-γ (IFNγ)-producing $CD4^+$ cells, and that the most overt effects of these components on tumor growth *in situ* are to provoke overt focal necroses and to decrease the stromal bed. Tumors growing in the presence of any of these components also show reduced expression of Rae-1, a ligand for the activating NK receptor, NKG2D. Collectively, these data illustrate which components of the αβ T cell response against SCC have protective potential, and indicate which aspects of tumor physiology may be most susceptible to their activities.

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In studies involving almost 4000 patients, collectively suffering from melanoma and a broad variety of carcinomas, a positive correlation has been made between survival and the presence of tumor-infiltrating lymphocytes (TIL) (reviewed by Dunn *et al*, 2002). Consistent with this, organ transplant recipients and other iatrogenically immunosuppressed patients demonstrate up to a 25-fold increased relative risk of cutaneous malignancy (Euvrard *et al*, 1997; Peto, 2001), with the greatest increase being in the development of squamous cell carcinoma (SCC) (Peto, 2001; Paul *et al*, 2003), that on average show greater progression and lethality (Veness *et al*, 1999; Martinez *et al*, 2003).

Support for a role of the adaptive immune response in regulating human cutaneous SCC is provided by TIL that include $TCR\alpha\beta^+$, $CD8^+$, $HLA\text{-}DR^+$ activated T cells that are cytotoxic toward autologous tumor cells directly *ex vivo* (Haeffner *et al*, 1997). At the same time, so-called keratoacanthomas, which represent well-differentiated SCCs with a high probability of involution (Hodak *et al*, 1993; Kerschmann *et al*, 1994), are substantially infiltrated by activated $CD4^+$ T cells when compared with invasive SCCs (Patel *et al*, 1994). Building on such results, several clinical approaches are being taken to enhance anti-tumor responses in patients (Banchereau *et al*, 2001; Dudley *et al*, 2002).

Nonetheless, the overt failure of the immune system to prevent tumor formation in outwardly healthy individuals and animals has often promoted the view that immunosurveillance of tumors is not generally applicable. An alternative interpretation that has recently gained experimental support is that the immune response to tumors, like that to infections, comprises a mixture of activities, the aggregate effects of which may not always be protective. Thus, Schreiber and colleagues (Siegel *et al*, 2000) showed that mice transgenic for an activated oncogene developed more, not less, tumors when pre-immunized with the oncogene, while Hanahan, Coussens and colleagues (Coussens *et al*, 2000; Daniel *et al*, 2003) showed that a bystander effect of anti-bacterial αβ T cells was to promote local inflammation that provoked tumor growth. Similarly, we recently showed that the αβ T cell response to chemically induced SCC contains within it an activity that can promote tumor progression (Girardi *et al*, 2003). Therefore, effective clinical manipulation of the anti-tumor immune response will require an improved understanding of which αβ T cell activities are protective and what their aggregate effects are on tumor growth.

Transplantable tumor systems represent a powerful means to establish such an understanding, because identical tumor inocula can be used to compare immunity in different groups of mice. To date, evidence from such experiments points to the combined activity of tumor-

Abbreviations: ELISpot, enzyme-linked immunoadsorption spot assay; IFNγ, interferon-γ; Rae-1, retinoic acid early-1; SLN, spleen and lymph node; TCR, T cell receptor

Sites of experiments: New Haven, Connecticut, USA, and London, UK

infiltrating $CD4^+$ and $CD8^+$ T cells, although the respective contributions of each subset appear to vary in different studies (reviewed in Blankenstein, 2003). Thus, while relatively few adoptively transferred tumor antigen-specific $CD8^+$ T cells were sufficient to eradicate established transplanted tumors (Hanson *et al*, 2000; Klein *et al*, 2003), production of interferon (IFN)γ by $CD4^+$ T cells appeared to be a critical effector mechanism against transplanted (Mumberg *et al*, 1999; Qin and Blankenstein, 2000) and chemically induced SCC (Kaplan *et al*, 1998; Qin *et al*, 2002). Moreover, recent data have highlighted that the establishment of effective $CD8^+$ T cell memory requires $CD4^+$ T cells (Shedlock and Shen, 2003; Sun and Bevan, 2003).

To examine this issue further, we have identified the components of the $\alpha\beta$ T cell response that are protective against the widely used transplantable SCC line, PDV (Fusenig *et al*, 1978; Diaz-Guerra *et al*, 1992). Moreover, we have examined how tumors growing *in situ* in the presence or absence of such components may differ from those growing in immunocompetent mice. Collectively, the data support the view that effective anti-tumor activities are an aggregate of $CD8^+$ and IFNγ-producing $CD4^+$ cells. The most overt effects of these components on tumor growth *in situ* are to provoke overt discrete areas of tumor necroses and to decrease the stromal bed. Tumors growing in the presence of any of these components also show reduced expression of Rae-1, a ligand for the activating NK receptor, NKG2D.

Results

$\alpha\beta$ T cell response to PDV tumor development in C57BL/6 mice Following intradermal injection of 10^6 PDV cells into each of the bilateral flanks (i.e., two sites per mouse) of 25 C57BL/6 mice, tumors developed in only 12.0% (3 of 25) of mice in only 10.0% (5 of 50) of sites. Histologic examination of the PDV tumors showed features diagnostic of well-differentiated cutaneous SCC (Fig 1*a*). When 12 of the mice

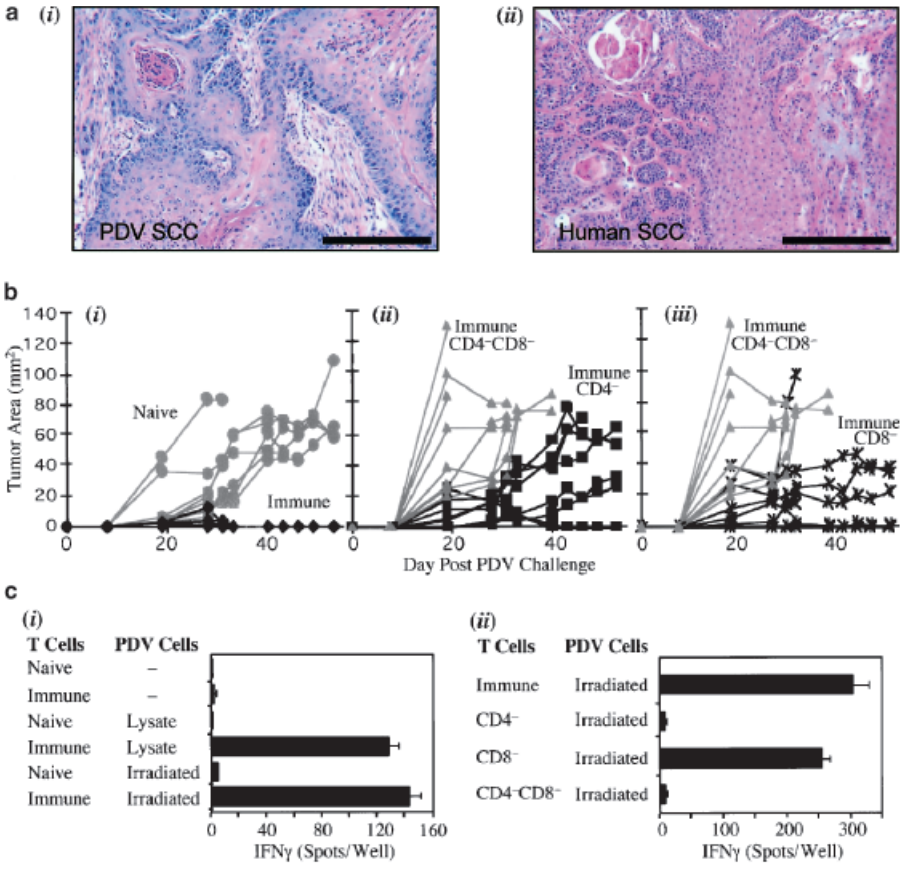

Figure 1
Both $CD4^+$ and $CD8^+$ T cells are required for optimal response to PDV SCC. (*a*, *panels i and ii*) Intradermal injection of C57BL/6 mice with 1×10^6 PDV cells may give rise to tumors that histologically resemble human SCC in that each exhibit infiltration of stroma by irregularly shaped, branching neoplastic lobules composed of keratinocytes with enlarged atypical nuclei and premature cornification (scale bar = 0.5 mm). (*b*) $TCR\beta^{-/-}$ recipients were challenged with PDV tumor cells 4 d after intravenous administration of unsorted or negatively depleted SLN cells from immunoprotected ("immune") or "naïve" B6 mice. The immune, but not naïve, cells transferred anti-tumor immunity as evidence by abrogation of tumor growth (*panel i*). If the cells from immune mice however were first depleted of $CD4^+$ cells ($CD4^-$, panel *ii*), $CD8^+$ cells ($CD8^-$, *panel iii*), or both ($CD4^-CD8^-$, depicted in both panels *ii* and *iii*), then tumor growth proceeded in most cases, demonstrating the dependence on both CD4 and CD8 T cells for optimal anti-tumor immunity. (*c*) SLN cells were plated at 1×10^5 cells per well, in duplicate. (C, panel *i*) Numbers of spots/well, indicative of IFNγ secretion, were markedly higher for SLN cells isolated from immune mice than for naïve C57BL/6 mice when in the presence of either irradiated cultured PDV cells ($p < 0.001$) or freeze–thaw lysates of freshly isolated PDV tumor cells ($p < 0.001$). Wells containing PDV cells or lysates alone showed no spots, and wells containing immune or naive T cells alone with 2 μg per mL concanavalin A showed >500 spots (data not shown). (*c*, *panel ii*) In an independent experiment, SLN cells from immune mice were plated at 1×10^5 cell equivalents per well, in duplicate. Wells containing $CD4^+$ T cells (i.e., unsorted and singly depleted of $CD8^+$ cells ($CD8^-$)) demonstrated the ability to produce more spots (indicative of IFNγ secretion) in the presence of irradiated PDV cells, when compared with wells not containing $CD4^+$ T cells (i.e., singly depleted of $CD4^+$ cells ($CD4^-$), $p < 0.001$; or doubly depleted of $CD4^+$ and $CD8^+$ cells ($CD4^-CD8^-$), $p < 0.001$). Error bars represent SEM.

Table I. Increased tumor-site incidence and decreased tumor latency in TCR mutant *versus* C57BL/6 mice following injection of PDV tumor cells

Mouse strain	Tumor-mouse incidence	Tumor-site incidence	Mean tumor latency[a]
C57BL/6	12.0% (3/25)	10.0% (5/50)	5.3 ± 2.4
TCR$\beta^{-/-}$	100.0% (15/15)	96.7% (29/30)	2.3 ± 0.2^{c}
TCR$\beta^{-/-}\delta^{-/-}$	100.0% (5/5)	100.0% (10/10)	2.2 ± 0.2^{c}

[a]time (in weeks) to tumor area $\geqslant$16 mm^2 post-injection of 1×10^6 PDV cells in mice that developed tumors.
[b]$p < 0.005$ *versus* C57BL/6.

Table II. SLN cells from immunoprotected C57BL/6 (B6) donors transfer protection to naïve TCR$\beta^{-/-}$ recipients

Donor	SLN[a] cell dose	Recipient	Tumor incidence
B6-naïve[b]	10×10^6	$\beta^{-/-}$	100% (4/4)
B6-immune[c]	10×10^6	$\beta^{-/-}$	0% (0/4)
B6-immune	1×10^6	$\beta^{-/-}$	33% (1/3)

[a]SLN = pooled spleen and lymph node cells (10×10^6 cell equivalents).
[b]Naïve = donors not previously challenged with PDV.
[c]Immune = immunoprotected donors showing no evidence of tumor at 8+ weeks post-challenge with PDV.

that failed to develop tumors at either site were re-challenged with a 10-fold larger inoculum of PDV cells, all were resistant to tumor formation, consistent with their development of immunological memory. By contrast, inoculation of 10^6 PDV cells per site led to tumor formation in 15 of 15 C57BL/6 TCR$\beta^{-/-}$ mice (that lack αβ T cells) and 5 of 5 C57BL/6 TCR$\beta^{-/-}\delta^{-/-}$ mice (that lack αβ and γδ T cells (Table I)). Furthermore, those relatively rare tumors that developed in immunocompetent C57BL/6 mice did so with a tumor latency (i.e., time from injection to development of palpable tumors) of 3–8 weeks (mean 5.3 weeks), whereas tumors in TCR$\beta^{-/-}$ and TCR$\beta^{-/-}\delta^{-/-}$ mice all developed in 2–3 weeks (mean 2.3 and 2.2 wk, respectively).

To confirm that the enhanced tumor susceptibility of TCR$\beta^{-/-}$ mice reflected the absence of αβ T cells, naïve TCR$\beta^{-/-}$ mice were intravenously reconstituted with SLN mononuclear cells from either naïve C57BL/6 mice or immunoprotected C57BL/6 mice (i.e., those demonstrating resistance to prior PDV challenge). Four days later, the mice were challenged with PDV tumor cells in the bilateral flanks (two sites per mouse; 1×10^6 PDV cells per site). Whereas 4 of 4 TCR$\beta^{-/-}$ mice receiving 10×10^6 SLN cells from naïve mice developed SCCs in 8/8 sites, no tumor developed in TCR$\beta^{-/-}$ mice receiving 10×10^6 SLN cells from immunoprotected mice (Table II). Moreover, some protection was afforded by adoptive transfer of as few as 1×10^6 SLN cells from immunoprotected mice (Table II). These data are consistent with a previous report that the T cell response to PDV is dominated by αβ T cells, rather than γδ T cells (Girardi *et al*, 2001).

To delineate which $\alpha\beta^+$ T cells were important for anti-tumor protection, TCR$\beta^{-/-}$ mice were inoculated with 10×10^6 "cell equivalents" (see *Materials and Methods*) of $CD4^+$, $CD8^+$, or $CD4^-CD8^-$ (double negative) SLN cells from immunoprotected donors, obtained by selective depletion of SLN cells using antibody-coated beads. The resistance of these mice to challenge 4 d later with PDV cells was compared with the full resistance of mice receiving unsorted SLN cells from immunoprotected mice, and the full susceptibility of mice receiving unsorted SLN cells from naïve mice (Fig 1*bi*). All mice receiving unsorted SLN cells from naïve mice or double negative SLN cells from immunoprotected donors succumbed to tumors (Fig 1*b*). Conversely, either $CD4^+$- or $CD8^+$-depleted SLN cells from immunoprotected donors conferred partial resistance, as evidenced by substantially diminished tumor growth rate (Fig 1*bi and ii*). Nonetheless, at 8 wk, the number of surviving mice, the tumor incidence for each group (four mice; eight sites per group), and the sizes of the tumors that developed collectively indicated that inoculation of immune SLN cells depleted of either $CD4^+$ or $CD8^+$ T cells provided markedly less protection than non-depleted inocula. In sum, while neither $CD4^+$ nor $CD8^+$ T cells are absolutely essential for tumor protection, both contribute substantially to the anti-tumor response.

Consistent with reports that $CD4^+$ T cells contribute to anti-tumor responses via IFNγ production (Mumberg *et al*, 1999), SLN cells from immunoprotected mice but not naïve mice were shown by ELISpot assay to produce IFNγ in response to irradiated PDV cells (Fig 1*c*). Moreover, by depleting SLN cells of $CD4^+$ or $CD8^+$ T cells, IFNγ production was attributable to $CD4^+$ T cells, not $CD8^+$ T cells.

T cell response to PDV tumor development in mutant mice To confirm independently the involvement of $CD4^+$ T cells, $CD8^+$ T cells, and IFNγ in the immune protection of C57BL/6 mice against PDV, tumor susceptibility was assessed in $CD4^{-/-}$ mice, $\beta_2m^{-/-}$ mice (that are devoid of $CD8^+$ T cells because such cells cannot be positively selected by a thymus lacking most MHC Class I molecules), and IFN$\gamma^{-/-}$ mice. Relative to normal C57BL/6 controls ($N = 15$ mice; 30 sites), all mutant mice ($N = 8$ mice; 16 sites) rapidly developed tumors (Fig 2). At 24 days post-challenge, tumor-site incidence was 93.8% in IFN$\gamma^{-/-}$ and $\beta_2m^{-/-}$ mice, and 80.0% in $CD4^{-/-}$ mice, compared with 20% in B6 controls and 100% in TCR$\beta^{-/-}$ mice. Moreover, the mean tumor size (scoring only sites with tumors) was significantly smaller in C57BL/6 mice (18.3 ± 5.1 mm^2) compared with $CD4^{-/-}$ (33.2 ± 4.7 mm^2), $\beta_2m^{-/-}$ (53.3 ± 12.2 mm^2), and IFN$\gamma^{-/-}$ mice (75.9 ± 9.8 mm^2) ($p < 0.002$ for C57BL/6 relative to IFN$\gamma^{-/-}$ mice).

Histologic differences in tumors growing in immunocompetent *versus* immunodeficient mice Tumors were dissected from immunocompetent C57BL/6 mice and from immunodeficient TCR$\beta^{-/-}$ or IFN$\gamma^{-/-}$ mice, H&E stained, and analyzed for morphologic differences by a pathologist

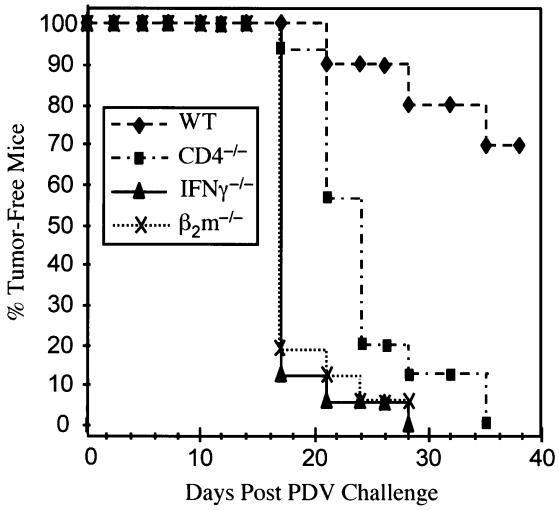

Figure 2
Analysis in immune knockout mice confirms dependence on multiple immune components for optimal anti-tumor immunity to PDV SCC. Relative to normal C57BL6 wild-type (WT) controls, all groups of mutant ($CD4^{-/-}$, $\beta_2m^{-/-}$, and $IFN\gamma^{-/-}$) mice developed tumors rapidly after intradermal challenge with PDV cells, confirming the dependence of an anti-tumor immune response on $CD4^+$ T cells, $CD8^+$ T cells, and IFNγ, respectively ($p < 0.01$ for WT *vs* $CD4^{-/-}$, $\beta_2m^{-/-}$, or $IFN\gamma^{-/-}$).

(EJG) in a blinded study. With 100% concordance, it was possible for tumors to be assigned either to fully immunocompetent hosts (C57BL/6), or to hosts that were partially ($IFN\gamma^{-/-}$) or fully ($TCR\beta^{-/-}$) immunodeficient. The assignment was based on the observation that tumors growing in $TCR\beta^{-/-}$ or $IFN\gamma^{-/-}$ mice consistently demonstrated a rich stroma and an absence of focal necrosis, relative to tumors growing in C57BL/6 mice (Fig 3). This was confirmed by the digital analysis of low-power images for the degree of necrotic or cystic changes, and the quantitative analysis of high-power images for the degree of stromal involvement.

NKG2D ligand expression by tumors growing in immunocompetent and immunodeficient mice One mechanism implicated in the anti-tumor response of cytolytic T cells (CTLs) is their engagement via the activating receptor, NKG2D, of ligands such as Rae-1 and MICA expressed by mouse and human tumor cells, respectively (Groh *et al*, 1998; Girardi *et al*, 2001). Indeed, introduction of Rae-1 into different tumor cell lines decreased their subsequent growth *in vivo* by increasing CTL and/or NK-cell-mediated rejection (Cerwenka *et al*, 2001; Diefenbach *et al*, 2001). The expression of ligands for NKG2D, however, has not previously been examined during the growth of tumors in immunocompetent *versus* immunodeficient mice. To test this, Rae-1 RNA expression was assessed by quantitative RT-PCR in three independent cultures of PDV cells, prior to inoculation, and in PDV tumors directly explanted from C57BL/6 or $TCR\beta^{-/-}$ mice, $CD4^{-/-}$, $\beta_2m^{-/-}$, and $IFN\gamma^{-/-}$ mice (Fig 4). Whereas independent cultures of PDV cells expressed comparable levels of Rae-1 (mean arbitrary units = 1.9), those levels were consistently and substantially reduced (mean arbitrary units = 0.2–0.5) in tumors harvested from mice with any one component of the anti-tumor αβ T cell response intact, i.e., $CD4^+$ T cells, $CD8^+$ T cells, and IFNγ. By contrast, Rae-1 levels were similarly reduced in only two of eight tumors of comparable stages of growth developing in $TCR\beta^{-/-}$ mice lacking αβ T cells. In the remaining six tumors from $TCR\beta^{-/-}$ mice, the mean expression level in arbitrary units is 1.3, closer to the levels expressed by PDV cells *in vitro*.

Discussion

For decades, organ transplant recipients treated with immunosuppressive medications have provided a living example of the importance of the immune response to skin cancer (Fetscher *et al*, 1997; Cohn, 1998; Galvao *et al*, 1998; Peto, 2001; Enzler *et al*, 2003). The markedly increased incidence of SCC development and metastasis in this population is consistent with various models of anti-tumor surveillance by lymphocytes, and supports a clinical strategy of using immunotherapy to combat tumor growth. Nevertheless, an aggregate protective effect of T cells on tumor development has not always been evident in animal models, and some immunotherapy protocols have shown mixed results in the clinic (Pardoll, 2002; Kalos, 2003). Whereas some investigators have used such data to dispute the concept of tumor immunosurveillance, several recent studies suggest that such data may be explained by a multi-component immune response to tumor growth, in which only some immunological activities are protective, while others *de facto* promote tumor growth (Siegel *et al*, 2000; Daniel *et al*, 2003; Girardi *et al*, 2003). In some respects, this may parallel the immune response to infection that is now appreciated to comprise both immunoprotective and immunosuppressive components (Belkaid *et al*, 2002). This perspective suggests that each of these components need to be better understood if a more reliable knowledge of tumor immunology is to be developed. To this end, this paper has sought to clarify which components of the αβ T cell response against SCC have protective potential, and to demonstrate which aspects of tumor physiology may be most susceptible to those activities.

$CD8^+$ cytotoxic T cells, $CD4^+$ T cells, and IFNγ have most commonly been implicated in tumor immunosurveillance both in humans and in animal studies (Soiffer *et al*, 2003; Patel *et al*, 1994; Hung *et al*, 1998; Kaplan *et al*, 1998; Marzo *et al*, 1999; Mumberg *et al*, 1999; Qin and Blankenstein, 2000; Blankenstein and Qin, 2003). Indeed, tumors have been reported to develop spontaneously in $IFN\gamma^{-/-}$ mice (Shankaran *et al*, 2001). The results in this study confirm the involvement of each of these components in the response to PDV tumors that develop with strong similarities to well-differentiated human SCC. This is consistent with the paradigm that tumors are unlikely to be vulnerable to a single immunological effector activity, e.g., cytolysis effects, anti-proliferative effects, or angiostatic effects. To investigate this further, some specific effects of the immune system on tumor growth were examined in this study.

We show that tumors growing in the absence of all αβ T cells, or in the absence of a single active immunological component (IFNγ) were strikingly different from those growing in their fully immunocompetent hosts. Consistently

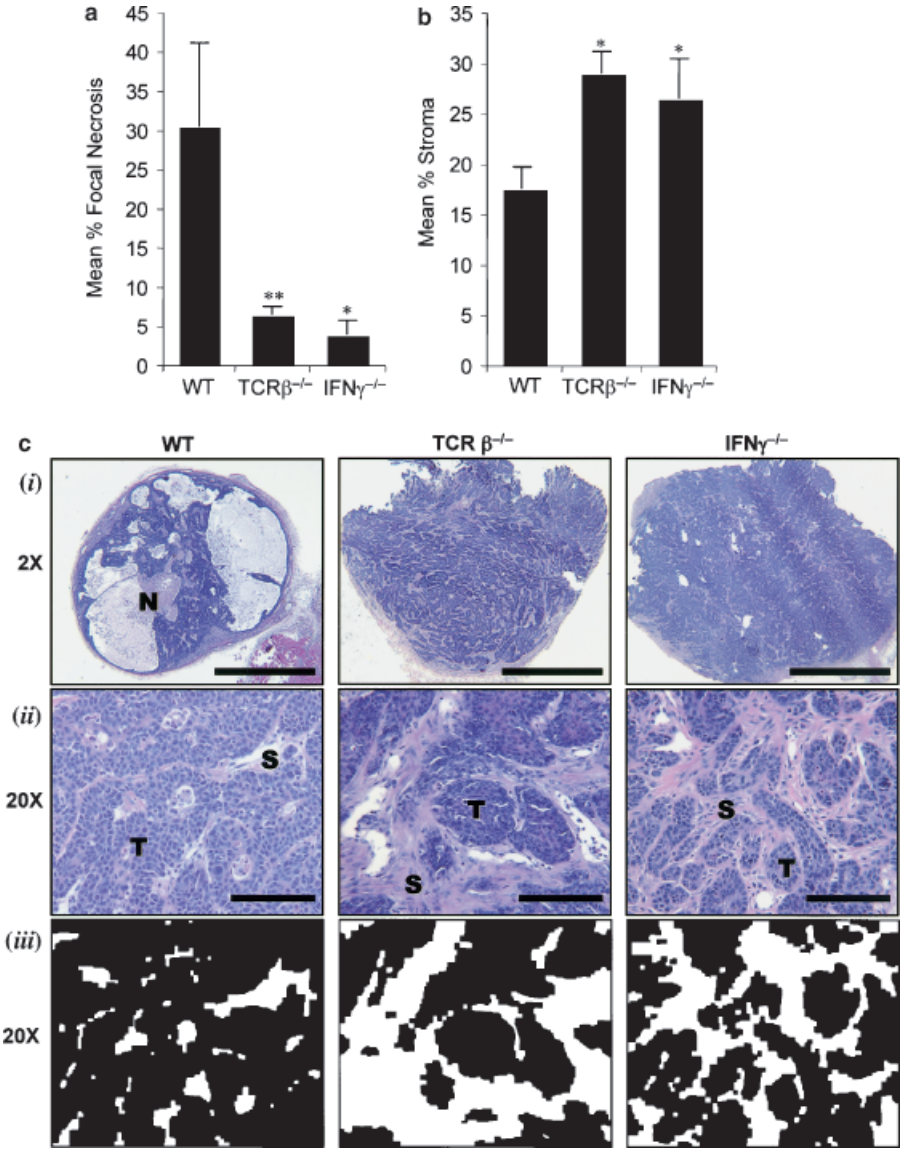

Figure 3
Tumors growing under immunocompetency display focal necrosis and a decreased stromal bed relative to tumors growing in the absence of αβ T cells or IFNγ. Focal necrosis (*a*) and tumor stroma area (*b*) were calculated as a percentage of tumor area after analysis of low-power and high-power histologic images of tumors growing in wild-type (WT), TCR$\beta^{-/-}$, and IFN$\gamma^{-/-}$ mice (error bars represent SEM; $^{*}p<0.05$, $^{**}p<0.01$). (*c*, *panels i and ii*) WT tumors typically exhibited foci of necrosis (**N**) and cystic changes at scanning magnification (representative section, 2 ×) and a paucity of stoma (**S**) at high magnification (representative section, 20 ×), relative to tumor cells (**T**). By contrast TCR$\beta^{-/-}$ and IFN$\gamma^{-/-}$ tumors showed little or no necrotic/cystic change (2 ×) and the presence of abundant stroma with prominent vasculature (20 ×) (scale bar = 1.0 mm for *panel i*, 0.025 mm for *panel ii*). (*c*, *panel iii*) For the quantification analysis shown in *a* and *b*, images were converted to gray scale. For low-power images, tumor regions were converted to black via thresholding in the curves function, and necrotic/cystic regions were similarly converted to white. For high-power images, only non-necrotic regions were utilized. Tumor regions were converted to black and the stomal/vascular compartment was converted to white. The percentage of black and of white pixels was then calculated for each image.

they displayed an enriched stroma and lacked the central necrosis characteristic of tumors that are under full immunosurveillance. Of note, the morphology of tumors growing in fully immunodeficient TCR$\beta^{-/-}$ mice was indistinguishable from that of tumors growing in partially immunodeficient IFN$\gamma^{-/-}$ mice. This suggests that IFNγ plays a major role in regulating the peri-tumor stroma, with consequences for the viability of cells in the core of the tumor. The observation that tumor cells themselves are more susceptible to surveillance if they express the IFNγ receptor (Shankaran *et al*, 2001; Basco *et al*, 2002) emphasizes the likelihood that the effects of IFNγ are pleiotropic (reviewed by Ikeda *et al*, 2002). However, among these effects of IFNγ, the data presented here support the possibility of a direct angiostatic effect on endothelial cells (Beatty and Patterson, 2000; Qin and Blankenstein, 2000). Thus, it was reported that the immune response of mice pre-immunized with irradiated tumor cells inhibited the angiogenesis of transplanted tumors via an IFNγ-dependent mechanism (Qin and Blankenstein, 2000). The potential of immunological effector mechanisms to target non-transformed stromal cells that support tumor development is important, because such cells, unlike malignant cells, do not have intrinsic chromosomal instability and are therefore unlikely to evade the immune surveillance. Indeed, angiostatic targeting of stromal cells may yet prove to be a common feature of immune responses against tumors, pathogens, and allografts.

By contrast to their indistinguishable morphology, tumors growing in TCR$\beta^{-/-}$ mice and IFN$\gamma^{-/-}$ mice show distinct expression patterns of the NKG2D ligand, Rae-1. Because tumors with similar morphologies (from TCR$\beta^{-/-}$ and IFN$\gamma^{-/-}$ mice) display different Rae-1 expression, while tumors with different morphologies (from C57BL/6 and IFN$\gamma^{-/-}$ mice) display similar Rae-1 expression, it is clear that the differences in Rae-1 expression do not segregate with the peri-tumor stroma. Most likely, these differences reflect an altered expression of Rae-1 on the tumor cells themselves; however, this would be difficult to establish experimentally since the harsh disaggregation techniques that would be required to separate tumor cells from supporting stroma are likely to perturb the expression of molecules known to be stress-responsive. Irrespective of the precise source of Rae-1 expression in the tumors, the pattern is consistent: all

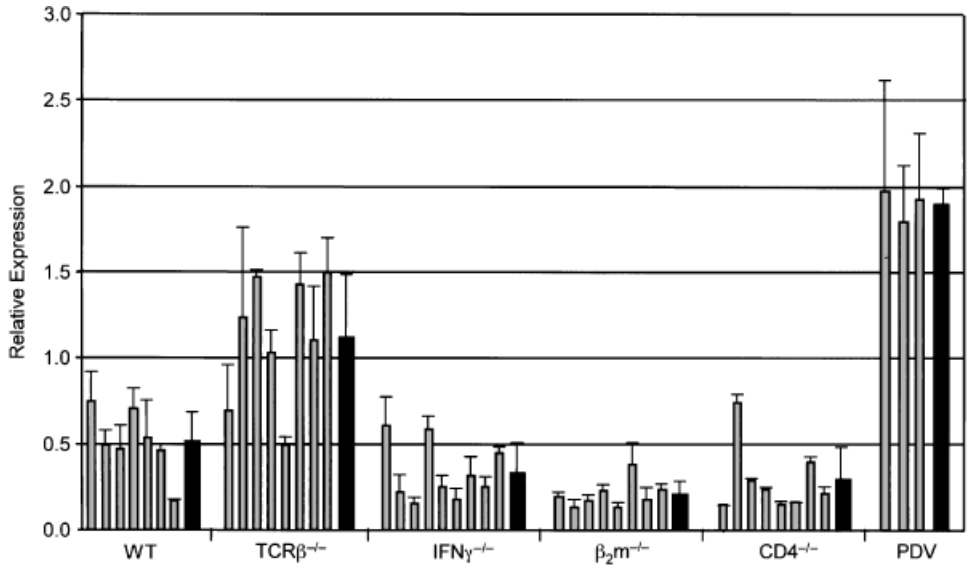

Figure 4
Expression of the NKG2d ligand Rae-1 in PDV tumors growing in normal *versus* immunodeficient mice. RNA expression was assessed by quantitative RT-PCR in three independent cultures of PDV cells and in PDV tumors directly explanted from wild-type (WT), TCR$\beta^{-/-}$, CD4$^{-/-}$, β_2m$^{-/-}$, or IFN$\gamma^{-/-}$ mice. Quantification of Rae-1 expression is shown for each individual tumor in gray bars, and the mean of all tumors within a group in black bars. Error bars represent the standard deviation of the mean. Tumors from TCR$\beta^{-/-}$ mice expressed significantly higher levels of Rae-1 than tumors from immunocompetent WT mice ($p < 0.002$).

tumors growing in hosts with any immunologically active component (C57BL/6, CD4$^{-/-}$, β_2m$^{-/-}$, IFN$\gamma^{-/-}$ mice) show substantially downregulated expression compared with PDV cells prior to inoculation. By contrast, there is only rare and less substantial downregulation in mice completely lacking all $\alpha\beta$ T cells. The reduced expression of the NKG2D ligand, Rae-1, in tumors developing in C57BL/6 mice is consistent with tumor editing imposed by CD8$^+$ T cell surveillance, and with the reports that experimentally imposed expression of Rae-1 in different tumor cell lines increased their rejection *in vivo* (Cerwenka *et al*, 2001; Diefenbach *et al*, 2001). The fact that Rae-1 expression was also reduced in tumors growing in β_2m$^{-/-}$ mice that lack CD8$^+$ CTLs is consistent with the fact, shown here, that the anti-tumor T cell response is multi-faceted. Thus, tumors still grow at a reduced rate in β_2m$^{-/-}$ mice relative to their growth in fully $\alpha\beta$ T cell-deficient mice, and the strong possibility exists that IL2-producing CD4 $\alpha\beta$ T cells promote the capacity of other cytolytic components (e.g., $\gamma\delta$ cells) to counter tumor growth.

The inverse correlation of Rae-1 expression and the host's immunocompetence is consistent with the prospect that by increasing NKG2D-ligand expression, immunological attention may be focussed onto a developing tumor. In a report that might seem to contradict the results reported here, sustained expression of MICA, a human MHC Class IB ligand for NKG2D, was observed to downregulate responding T cells or NK cells (Groh *et al*, 2002). A notable feature however of that study was the evidence that sustained MICA expression correlated with the capacity of tumors to secrete the protein, which thereby can act as a molecular decoy, binding NKG2D with no stimulatory consequence. Thus, rather than being contradictory, both sets of experiments highlight the importance of NKG2D ligands in characterizing tumor growth. By further analysis of the growth of carcinomas in immunocompetent and immunodeficient hosts, as described here, it should be possible to identify the means and kinetics by which NKG2D ligand expression can be regulated within tumor masses developing *in vivo*, and whether or not the modulation of NKG2D ligand expression is of equivalent significance at different stages of tumor development. One would expect that such information might be useful for the further development and application of improved immunotherapy modalities.

Materials and Methods

Animals TCR$\beta^{-/-}$ (Mombaerts *et al*, 1992), TCR$\beta^{-/-}\delta^{-/-}$ (Mombaerts *et al*, 1992; Itohara *et al*, 1993), IFN$\gamma^{-/-}$ (Dalton *et al*, 1993), β_2-microglobulin (β_2m)$^{-/-}$ (Koller *et al*, 1990), and CD4$^{-/-}$ (McCarrick *et al*, 1993) mice (previously backcrossed 10+ generations onto C57BL/6 (B6)), and wild-type B6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained as local colonies under pathogen-free conditions in micro-isolator cages with autoclaved food. All of the *in vivo* studies performed were approved by the Yale Animal Use and Care Committee.

Cell culture The PDV keratinocyte tumor cell line, which grows as an adherent squamoid monolayer in culture, was originally derived by single exposure of normal keratinocytes to the carcinogen 7,12-dimethyl-benzanthracene (DMBA). Molecular analysis has identified a mutation in codon 61 of the H-ras gene (Diaz-Guerra *et al*, 1992). Intradermal injection of 1×10^6 PDV cells into C57BL/6 mice had previously been demonstrated to result in tumor development in approximately 10%–20% of injection sites (Caulin *et al*, 1993; Girardi *et al*, 2001). PDV cells were maintained in complete RPMI (CRPMI; RPMI 1640; Invitrogen, Palo Alto, California) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 μM non-essential amino acids, 1 mM sodium pyruvate, 40 M Hepes buffer, 5×10^{-5} M 2-mercaptoethanol, and antibiotics.

Isolation of spleen and lymph node (SLN) cells Single-cell suspensions were prepared as previously described (Girardi *et al*, 1995). Briefly, SLN cells were gently pressed between the frosted edges of two glass slides, releasing the cells into ice-cold Hank's balanced salt solution (HBSS) containing 4 mM Hepes buffer and

antibiotics. Cells were filtered through nylon mesh (Nytex cloth 88/42; Tetko, Elmsford, New York) to remove debris and to give single-cell suspensions. SLN cells were washed twice with HBSS before and after lysis of red blood cells using Red Blood Cell Lysis Buffer (Sigma, St Louis, Missouri) according to the manufacturer's directions. Cells were washed, counted, and resuspended at appropriate concentrations in phosphate-buffered saline (PBS) (Invitrogen) for adoptive transfer, HBSS for antibody depletion, or CRPMI for ELISpot assay. For adoptive transfer experiments, sorted or unsorted SLN cells were injected in 100 μL PBS at the indicated concentrations into the tail vein of recipient mice.

PDV tumor challenge Cultured PDV cells were trypsinized (Caulin *et al*, 1993) and washed in PBS three times, counted, and resuspended in PBS before intradermal injection (i.e., raising a bleb) of 1×10^6 cells per site via a 25-gauge needle. Mice were observed approximately weekly for palpable tumors, which were measured in two dimensions to determine tumor area. Mice were euthanized when tumor size exceeded 100 mm^2, or earlier if tumor ulceration developed.

T cell subset depletion Negative depletion of T cell subsets was performed using Dynal iron bead-conjugated anti-$CD4^+$ and $CD8^+$ antibodies (Dynabeads, Dynal Biotech Inc., Lake Sucess, NY), according to the manufacturer's specifications. Briefly, the desired amount of Dynabeads was transferred to a test tube that was placed on a Dynal MPC magnet, and the fluid was removed leaving the beads intact. The vial was removed from the magnet and the beads were resuspended in 2 mL of HBSS, washed once more, resuspended in HBSS, and mixed with lymphocytes at a ratio of 4×10^7 beads per 1×10^7 cells, briefly vortexed, and incubated for 20 min at 4°C with gentle tilting and rotation on a Dynal MX1 apparatus. For doubly depleted cells, both $CD4^+$ and $CD8^+$ Dynabeads were added. After incubation, the tube was placed in the Dynal MPC magnet for 2 min before the $CD4^+$- and/or $CD8^+$- depleted supernatant cell suspension was removed, centrifuged, and resuspended in PBS prior to intravenous injection or ELISpot assay. For both the adoptive transfer and ELISpot assay utilizing CD4- and/or CD8-depleted populations, "cell equivalents" were utilized, as previously described (Gocinski and Tigelaar, 1990).

ELISpot assay ELISpot was carried out as previously described (White *et al*, 1999), with modifications noted here. Briefly, wells of MultiScreen 96-well plates (Millipore Billerica, MA) were coated with purified rat anti-mouse IFNγ monoclonal antibody (mAb) (Pharmingen, #R4-6A2) (BD Sciences, San Diego, CA) at 10 μg per mL in PBS (75 mL per well) at 4°C overnight, blocked with 5% FBS in PBS at room temperature (RT) for 2 h, and then rinsed 3 times with PBS. SLN cells were plated in the presence or absence of γ-irradiated (3000 rad) PDV target cells in a final volume of 200 μL per well in CRPMI, and incubated at 37°C and 5% CO_2 overnight. Wells were rinsed 5 times with 0.05% Tween-20 in PBS (PBS-T) before the addition of 100 μL of 5 μg per mL biotinylated rat anti-mouse IFNγ mAb (Pharmingen, XMG1.2) in 5% FBS in PBS-T at RT for 2 h. Wells were rinsed extensively with PBS-T, and then incubated with peroxidase-labeled streptavidin (KPL) at 1/800 dilution in PBS-T containing 5% FBS at RT for 30 min. Spots were developed using Sigma 3-amino-9-ethylcarbazole (AEC) substrate with dimethylformamide (DMF) and 30% hydrogen peroxide for up to 15 min, and visualized with an inverted-light microscope for counting.

Quantitation of RNA expression Tumors were isolated, dissected, and snap-frozen in LN_2 before homogenization in TRIzol (Invitrogen, Carlsbad, California) and total RNA extraction according to the manufacturer's instructions. RNAs were also prepared from three independent cultures of PDV. For quantitation of Rae-1 expression, cDNAs were prepared using Superscript II RT polymerase (Invitrogen), and PCRs were run in the ABI 7700 (Perkin-Elmer, Boston, Massachussets) using the following primers and 5′ FAM/3′ TAMRA-labeled probes:

Rae-1	sense:	5′-ATGGCCAAGGCAGCAGTG-3′
Rae-1	antisense:	5′-GGTCAAGTTGCACCTAAGAGAGTG-3′
Rae-1	probe:	5′-AACAGCTTCTGAATCATAAAATGATGGCG-CTT-3′
β-actin	sense:	5′-GTCATCACTATTGGCAACGAG-3′
β-actin	antisense:	5′-CACTGTGTTGGCATAGAGGTC-3′
β-actin	probe:	5′-CCATCATGAAGTGTGACGTTGACA-3′.

Histologic analysis Tumors from each animal were fixed in formalin, embedded in paraffin, and standard 5 μm histologic sections were prepared. Slides were routinely stained with hematoxylin and eosin (H&E) and with anti-CD31 (ABP method) (DAKO Cytomation, Carpenteria, California). One of the authors (EJG), a pathologist, examined each section blindly and performed quantitative analysis of tumor necrosis and cystic change *versus* stroma and vascular components. Low ($2 \times$)- and high ($20 \times$)-magnification digital photographs of H&E-stained sections of each tumor were analyzed quantitatively by the same pathologist via Adobe Photoshop 7.0 for Macintosh (Adobe Systems Incorporated, San Jose, CA). Photos were converted to gray scale. For low-power images, tumor regions were converted to black via thresholding in the curves function, and necrotic and/or cystic regions were similarly converted to white. For high-power images, only non-necrotic regions were utilized. Tumor regions were converted to black and the stromal/vascular compartment was converted to white. The percentage of black and of white pixels was then calculated for each image.

Statistics Statistical significance was evaluated by the two-tailed, unpaired Student's *t* test, or non-parametric analysis if standard deviations were significantly different between the two compared groups.

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