

## **Suppression of Lymphoma and Epithelial Malignancies Effected by Interferon $\gamma$**

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### **Abstract**

The immunosurveillance of transformed cells by the immune system remains one of the most controversial and poorly understood areas of immunity. Gene-targeted mice have greatly aided our understanding of the key effector molecules in tumor immunity. Herein, we describe spontaneous tumor development in gene-targeted mice lacking interferon (IFN)- $\gamma$  and/or perforin (pfp), or the immunoregulatory cytokines, interleukin (IL)-12, IL-18, and tumor necrosis factor (TNF). Both IFN- $\gamma$  and pfp were critical for suppression of lymphomagenesis, however the level of protection afforded by IFN- $\gamma$  was strain specific. Lymphomas arising in IFN- $\gamma$ -deficient mice were very nonimmunogenic compared with those derived from pfp-deficient mice, suggesting a comparatively weaker immunoselection pressure by IFN- $\gamma$ . Single loss of IL-12, IL-18, or TNF was not sufficient for spontaneous tumor development. A significant incidence of late onset adenocarcinoma observed in both IFN- $\gamma$ - and pfp-deficient mice indicated that some epithelial tissues were also subject to immunosurveillance.

Key words: immunosurveillance • effector • interferon • lymphoma • adenocarcinoma

### **Introduction**

Although tumor immunosurveillance was first hypothesized more than four decades ago, the observed absence of spontaneous tumors in several immune-compromised mouse strains initially dampened enthusiasm for this idea. mAbs specific for immunoregulators have sometimes proven useful in establishing the importance of such molecules in immunity against experimental tumors (1–3). Production of gene-targeted mice for specific effector molecules has greatly aided this endeavor (4–14). Nevertheless, experimental tumor models examined to date have provided limited information on tumor initiation and development. IFN- $\gamma$  (15) and perforin (pfp; references 5, 14, and 16), key molecules of both the innate and adaptive immune systems, contribute to tumor prevention in mice treated with the chemical carcinogen, methylcholanthrene (MCA), or in mice deficient for p53 expression. In MCA- (10) and other carcinogen-induced cutaneous malignancy models (17), the importance of antitumor immunity mediated by NK cells, NK T cells, and  $\gamma\delta^+$  T cells has also been illustrated. How-

ever, in most of these models tumor development involves the transformation of many cells at one time and only a defined spectrum of tumors (lymphomas and cutaneous malignancies) is observed. An alternative model of tumor induction is that spontaneous tumors can arise from a single cell of any tissue that has acquired the appropriate genetic mutations with age in any given environment. Herein, that environment has been controlled, and spontaneous tumor initiation compared in WT and gene-targeted mice of the same genetic background to illustrate the key role pfp and IFN- $\gamma$  play in tumor immunosurveillance of lymphoma and lung adenocarcinoma.

### **Materials and Methods**

*Mice.* Inbred C57BL/6J and BALB/c WT mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. The following gene-targeted mice were bred and maintained at the Austin Research Institute Biological Research Laboratories, Heidelberg, Australia and the Peter MacCallum Cancer Institute, East Melbourne, Australia: C57BL/6J pfp-deficient (B6 pfp<sup>-/-</sup>) (targeted in C57BL/6J ES cells and provided by D. Kagi [Amgen Institute, Toronto, Canada]; reference 18); C57BL/6J TNF-deficient (B6 TNF<sup>-/-</sup>) (targeted in C57BL/6J ES cells [reference 19] and provided by Dr.

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J.D. Sedgwick and originally from the Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia); C57BL/6 IFN- $\gamma$ -deficient (B6 IFN- $\gamma^{-/-}$ )(backcrossed to C57BL/6J for 10 generations from Genentech, Inc.; reference 20); C57BL/6 IL-12p40-deficient (B6 IL-12 $^{-/-}$ )(backcrossed to C57BL/6J for 10 generations from Hoffmann-La Roche; reference 21); C57BL/6 IL-18-deficient (B6 IL-18 $^{-/-}$ ) (backcrossed to C57BL/6J for eight generations and provided by Dr. S. Akira, Osaka University, Japan; reference 22); BALB/c pfp $^{-/-}$  (backcrossed to BALB/c for eight generations using microsatellite mapping), BALB/c IFN- $\gamma^{-/-}$  (backcrossed to BALB/c for 10 generations from Genentech, Inc., reference 20); and BALB/c pfp $^{-/-}$  IFN- $\gamma^{-/-}$ . F1 heterozygote progeny of some gene-targeted mice and their corresponding WT were bred as follows: (B6  $\times$  B6 IFN- $\gamma^{-/-}$ )F1; (BALB/c  $\times$  BALB/c IFN- $\gamma^{-/-}$ )F1; and (BALB/c  $\times$  BALB/c pfp $^{-/-}$ )F1. All aging mice were routinely screened for viruses, parasites, and other microbes and tested negative over the entire course of the experiment. Mice within the study were monitored for health and weighed twice weekly. Any mouse with an abnormality (palpable mass, abdominal distension, weight loss >10%, ruffled fur) was killed, its age recorded, and a post-mortem performed. Mean lifespan  $\pm$  SEM was calculated and probability of significance determined using a Mann-Whitney Rank Sum U-test. The significance of proportions of tumors and, in particular, disseminated lymphomas, was determined by a Fisher's exact test.

**Histopathology and Surface Phenotyping of Tumors.** A full autopsy was performed at sacrifice and tumor (macroscopically detected), spleen, liver, thymus, and lymph nodes were routinely examined by histology after fixing these tissues in formalin and on occasions also fresh frozen. The preparation and staining of sections for histology were performed by the Department of Anatomical Pathology, Austin and Repatriation Medical Centre, Heidelberg, Australia. Lymphomas from mice were also assessed for surface phenotype by multiparameter flow cytometric analysis. The following reagents were used: anti- $\alpha\beta$  TCR-APC (H57-597; BD PharMingen); NK1.1-PE (PK136; BD PharMingen); CD4-FITC (CT4; Caltag Laboratories); CD8 $\alpha$ -APC (53.6.7; BD PharMingen); CD8 $\beta$ -biotin (53.5.8; BD PharMingen); CD11c-FITC (HL3; BD PharMingen); B220-PE (RA3-6B2; Caltag Laboratories); Thy-1-PE (30-H12; BD PharMingen); CD45.2-FITC (104; BD PharMingen);  $\gamma\delta$  TCR-biotin (clone GL3; BD PharMingen); Mac-1-biotin (M170; Caltag Laboratories); goat anti-mouse Ig-FITC (Silenus Laboratories); and streptavidin-PerCP (BD PharMingen). Anti-Fc receptor (2.4G2) was used to prevent nonspecific binding by mAb. Staining was performed in PBS with 5% FCS and 0.02% sodium azide on ice. Fresh splenocytes from 6-wk-old B6 or BALB/c mice were always used as labeling controls. Analysis was performed on a FACScalibur<sup>TM</sup> using CELLQuest<sup>TM</sup> software (Becton Dickinson).

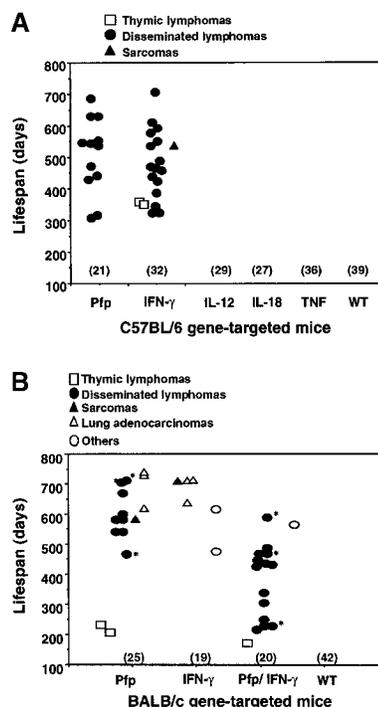
**Tumor Transplantation Experiments.** A number of disseminated lymphomas were transplanted directly from B6.pfp $^{-/-}$ , BALB/c pfp $^{-/-}$ , or B6 IFN- $\gamma^{-/-}$  mice into WT mice or mice of the same genotype. Two representative experiments are shown using a B cell lymphoma from B6.pfp $^{-/-}$  mice, PNK7 (B220<sup>+</sup>Ig<sup>+</sup>TCR- $\alpha\beta$ <sup>-</sup>), and a T cell lymphoma from B6 IFN- $\gamma^{-/-}$  mice, BG18 (CD8- $\alpha\beta$ <sup>+</sup>CD4<sup>-</sup>TCR- $\alpha\beta$ <sup>+</sup>Ig<sup>-</sup>). Groups of five WT or gene-targeted mice were injected intraperitoneally with increasing numbers of lymphoma cells and observed daily for tumor growth for >100 d.

**Online Supplemental Materials.** The supplemental figures are a (Fig. S1) histology of spontaneous neoplasia in immunodeficient mice and a (Fig. S2) flow cytometric analysis of lymphomas arising

in BALB pfp $^{-/-}$  and B6 pfp $^{-/-}$  mice. These can be found at <http://www.jem.org/cgi/content/full/jem.20020063/DC1> or from the authors by request.

## Results and Discussion

We undertook to monitor spontaneous tumor development in WT C57BL/6 (B6) and BALB/c mice or those that were deficient in IFN- $\gamma$  and/or pfp. B6 mice deficient in IL-12, IL-18, or TNF were also examined, as all of these cytokines have been shown to regulate the expression of IFN- $\gamma$  and other effector molecules in lymphocytes (23–27). B6 IFN- $\gamma^{-/-}$  mice developed disseminated lymphomas (16/32 (50%) and mean lifespan  $491 \pm 104$  d) (Fig. 1 A). A few mice developed thymic lymphomas ( $n = 2$ ) or sarcoma ( $n = 1$ ). Like WT B6 mice (0/39 mice), B6 mice that were deficient in TNF (0/36), IL-12 (0/29), or IL-18 (0/27) did not develop any tumors over the same observation period (Fig. 1 A). These data illustrate spontaneous lymphoma formation in IFN- $\gamma$ -deficient mice and suggest that more than one of the upstream mediators of IFN- $\gamma$  or an alternative cytokine must be required to regulate the



**Figure 1.** Pfp and IFN- $\gamma$  protect mice from spontaneous lymphoma. The appearance of tumors was recorded in mice of (A) C57BL/6 and (B) BALB/c backgrounds as indicated. Groups of mice (number in parentheses) were evaluated on a weekly basis and, when moribund, tumor type (white squares, thymic lymphoma; black squares, disseminated lymphoma; black triangles, sarcoma; white triangles, lung adenocarcinoma; and white circles, other tumors) recorded against the age at the time of death/autopsy (in days). \*, disseminated lymphomas of histiocyte morphology. No tumors were observed in heterozygote control groups of (B6  $\times$  B6 IFN- $\gamma^{-/-}$ )F1 ( $n = 17$ ), (BALB/c  $\times$  BALB/c IFN- $\gamma^{-/-}$ )F1 ( $n = 12$ ), and (BALB/c  $\times$  BALB/c pfp $^{-/-}$ )F1 ( $n = 18$ ) mice over a 750-d period. A small number of the oldest surviving BALB/c IFN- $\gamma^{-/-}$  mice (700–750 d) also developed nonmalignant mucosal hamartomas in the stomach.

protective effects of IFN- $\gamma$ . Consistent with our previous study (16), B6 pfp<sup>-/-</sup> mice died from aggressive disseminated lymphomas affecting the spleen, liver, and lymph nodes from 300 d onwards, with 57% (12/21) succumbing by the end of the experiment (mean lifespan = 510  $\pm$  119 d; Fig. 1 A).

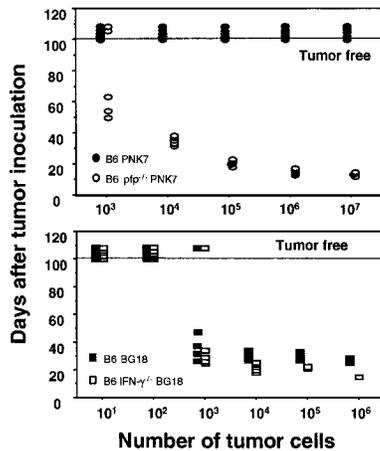
Of great interest was a considerably distinct pattern of tumor development observed in similar gene-targeted mice on a BALB/c background (Fig. 1 B). In striking contrast to B6 IFN- $\gamma$ <sup>-/-</sup> mice, BALB/c IFN- $\gamma$ <sup>-/-</sup> mice did not develop disseminated lymphoma (0/19,  $P$  = 0.0001). The finding that IFN- $\gamma$  was not essential for host protection from this type of tumor in BALB/c mice illustrated that strain-specific genetic factors can influence the nature of the immune response to these spontaneous lymphomas. By contrast, pfp was critical in host protection from disseminated lymphoma in both strains (Fig. 1 A versus Fig. 1 B). 9 of 25 BALB/c pfp<sup>-/-</sup> mice developed disseminated lymphoma (mean lifespan = 597  $\pm$  82 d), and a few mice developed thymic lymphomas ( $n$  = 2) or sarcoma ( $n$  = 1; Fig. 1 B). The earlier onset (mean lifespan = 368  $\pm$  120 d) ( $P$  < 0.0001, compared with pfp<sup>-/-</sup> mice) and greater frequency (14/20) of disseminated lymphoma in BALB/c mice deficient in both pfp and IFN- $\gamma$ , revealed that in the absence of pfp, IFN- $\gamma$  did play an important role in delaying the development of disseminated lymphoma (Fig. 1 B). These data supported a general role for IFN- $\gamma$  in protection from spontaneous lymphoma, but suggested that the relative role of IFN- $\gamma$  was strain specific.

Lymphocyte-mediated immunosurveillance of epithelial malignancy has never been demonstrated, except where viral infection may contribute to oncogenesis (e.g., EBV, HPV transplant recipients) or when carcinogens were also administered, and we therefore felt the development of spontaneous epithelial tumors in gene-targeted mice warranted particular attention. While IFN- $\gamma$  and pfp were especially important for the control of disseminated lymphoma, a significant incidence of lung adenocarcinoma was detected in BALB/c IFN- $\gamma$ <sup>-/-</sup> mice (3/19,  $P$  = 0.0269) and BALB/c pfp<sup>-/-</sup> mice (3/25,  $P$  = 0.048) (Fig. 1). These data directly demonstrated that IFN- $\gamma$  and pfp suppressed some epithelial malignancies. Notably, it is relevant that the onset of lung adenocarcinomas was very late in BALB/c IFN- $\gamma$ <sup>-/-</sup> (681  $\pm$  43 d) and BALB/c pfp<sup>-/-</sup> (693  $\pm$  67 d) mice. Indeed, the development of all tumors, including disseminated lymphomas was significantly delayed in BALB/c mice compared with B6 mice. Far fewer BALB/c pfp<sup>-/-</sup> IFN- $\gamma$ <sup>-/-</sup>, B6 IFN- $\gamma$ <sup>-/-</sup>, and B6 pfp<sup>-/-</sup> mice remained alive (free of lymphoma) over the first 600 d of life and therefore larger numbers of these will likely be required to observe adenocarcinomas. F1 progeny of gene-null mice and WT mice lacking either one allele of pfp or IFN- $\gamma$  did not develop any spontaneous tumors (Fig. 1). Determining whether the immune system controls other epithelial malignancies is an important issue for future long-term studies and only a similar study of far larger groups of mice ( $\geq$ 100) will reveal whether significant numbers of other epithelial malignancies emerge in IFN- $\gamma$ <sup>-/-</sup> and pfp<sup>-/-</sup> mice.

6 of the 16 lymphomas arising in B6 IFN- $\gamma$ <sup>-/-</sup> mice were analyzed for phenotype and while five were TCR- $\alpha\beta$ <sup>+</sup>, no unique lymphoma phenotype was apparent (unpublished data). Several previous studies have indicated that T cell death after antigen-driven expansion may be regulated by IFN- $\gamma$  (28, 29), and therefore possibly disrupted homeostasis and failed immunosurveillance by IFN- $\gamma$  loss, may be the cause of T cell lymphoma in B6 IFN- $\gamma$ <sup>-/-</sup> mice. All of the disseminated lymphomas in B6 pfp<sup>-/-</sup> mice were of B cell origin (B220<sup>+</sup> sIg<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup>TCR<sup>-</sup>), or plasmacytomas (B220<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> TCR<sup>-</sup> sIg<sup>low</sup>; also defined histologically, unpublished data). Our previous study of pfp<sup>-/-</sup> mice with additional p53 loss indicated a majority of lymphomas of B cell origin, however some p53<sup>+/-</sup>pfp<sup>-/-</sup> mice also developed lymphomas of non-B cell origin (16). One explanation for the development of B cell lymphomas in pfp<sup>-/-</sup> mice is the recognized role for pfp in controlling the survival of some APCs, like B cells (30, 31). However, it remains unclear why only lymphomas of B cell origin developed in B6 pfp<sup>-/-</sup> mice when enhanced expansions of CD8<sup>+</sup> T cells were observed in these mice, particularly after challenge with foreign antigens (e.g., viral or bacterial infection; references 28, and 32–34). Most of the lymphomas in B6 pfp<sup>-/-</sup> and B6 IFN- $\gamma$ <sup>-/-</sup> mice were diffuse large cell lymphomas (see Online Supplemental Materials).

Flow cytometric analysis of disseminated lymphomas in BALB/c pfp<sup>-/-</sup> and BALB/c pfp<sup>-/-</sup>IFN- $\gamma$ <sup>-/-</sup> mice revealed that all were also B220<sup>+</sup> sIg<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> TCR<sup>-</sup> (unpublished data). Importantly, these data supported a role for IFN- $\gamma$  in also suppressing the earlier onset of B cell lymphomas. Interestingly, in the BALB/c strain, a small number of the lymphomas arising in pfp<sup>-/-</sup> and pfp<sup>-/-</sup>IFN- $\gamma$ <sup>-/-</sup> mice showed an unusual histiocytic appearance (Fig. 1, asterisk) with a pale eosinophilic cytoplasm. These tumors additionally expressed CD11c and Mac-1 antigens (see Online Supplemental Materials), but did not all express other markers of B1 cells (e.g., CD5, unpublished data). Of note, this type of histopathology occurs only rarely in humans, and interestingly the accumulations of activated histiocytes (macrophages) observed in pfp-deficient humans displaying familial hemophagocytic lymphohistiocytosis (35) are one example. All the lung adenocarcinomas in both BALB/c pfp<sup>-/-</sup> and BALB/c IFN- $\gamma$ <sup>-/-</sup> mice were well-differentiated papillary adenocarcinomas (see Online Supplemental Materials).

The malignancy of primary tumors arising was additionally confirmed by secondary transfer into mice of the same strain. Two representative experiments of >10 using different disseminated lymphomas transferred from either B6 pfp<sup>-/-</sup> or B6 IFN- $\gamma$ <sup>-/-</sup> mice are depicted in Fig. 2. The transfer of three different T cell lymphomas from B6 IFN- $\gamma$ <sup>-/-</sup> mice (one shown) demonstrated that these tumors grew at a similar rate in B6 IFN- $\gamma$ <sup>-/-</sup> and B6 WT mice (Fig. 2). By contrast, and in concert with our previous data (16), all B cell lymphomas from B6 pfp<sup>-/-</sup> mice grew at low cell numbers in B6 pfp<sup>-/-</sup> mice, but were avidly rejected when transferred into B6 WT mice (Fig. 2).



**Figure 2.** Distinct behavior of lymphomas arising in B6  $pfp^{-/-}$  mice and B6  $IFN-\gamma^{-/-}$  mice. Primary lymphomas arising in B6  $pfp^{-/-}$  (top) and B6  $IFN-\gamma^{-/-}$  (bottom) were secondarily transplanted intraperitoneally ( $10^1$ – $10^7$  cells in 0.2 ml PBS, as indicated) into groups of five untreated B6 WT (black circles or squares), B6  $pfp^{-/-}$  (white circles) or B6  $IFN-\gamma^{-/-}$  (white squares) mice. Mice were monitored for 100 d and each symbol depicts an individual mouse. Tumor-free mice are indicated above the horizontal line. The results are representative of seven primary lymphomas and three primary lymphomas transplanted from B6  $pfp^{-/-}$  and B6  $IFN-\gamma^{-/-}$  mice, respectively. Top, PNK-7 (B cell lymphoma from B6  $pfp^{-/-}$  mouse); bottom, BG18 (T cell lymphoma from B6  $IFN-\gamma^{-/-}$  mouse).

Thus far, all lymphomas of B cell origin arising in  $pfp^{-/-}$  mice, including five described herein, were rejected in WT mice by  $CD8^+$  T cells (reference 16 and unpublished data) and in this study the donor tumor cells and recipient mice were genetically matched as closely as possible. Similar experiments with three different B cell lymphomas from BALB/c  $pfp^{-/-}$  mice have also demonstrated rejection in BALB/c WT mice and (BALB/c  $\times$  BALB/c  $pfp^{-/-}$ )F1 heterozygous mice, but not BALB/c  $pfp^{-/-}$  mice (unpublished data). In concert with our previous lymphoma transfer studies (16), it is likely that the lymphomas of B cell origin are generally being detected by  $CD8^+$  T cells independently of any alloantigens potentially expressed by the tumor. It is probable that the lack of detectable immunogenicity of lymphomas from  $IFN-\gamma^{-/-}$  mice indicates an important functional distinction between the activities of  $IFN-\gamma$  and  $pfp$  in eliminating potentially transformed cells. Consistent with the immune system functioning as a tumor-suppressor system, our results indicated that the immunoselection pressure of  $pfp$  on tumor cells was strong. Lymphomas from  $pfp$ -deficient mice have emerged in the absence of this significant immunoselection pressure, and they are avidly rejected in WT mice upon transfer. Immunoselection by  $IFN-\gamma$  appears far weaker from our spontaneous lymphoma transplant data, however it has been demonstrated in an experimental system using MCA fibrosarcomas arising in  $IFN-\gamma$  receptor-deficient mice (36).

Previous studies have demonstrated that T cells (2), NK T cells (37), and NK cells (37) can all produce  $IFN-\gamma$  with

antitumor activity. It remains unclear which type of lymphocytes use  $IFN-\gamma$  to control spontaneous lymphomas or lung adenocarcinomas, and this issue is very difficult to address without mice conditionally deficient (e.g., NK cell, NK T cell, or T cell) in  $IFN-\gamma$ . Defining exactly how a pleiotropic cytokine like  $IFN-\gamma$  mediates host protection from spontaneous tumors will also be a challenging goal for future investigation given that the lymphomas arising in B6  $IFN-\gamma^{-/-}$  mice appear nonimmunogenic when transferred into WT mice. Many potential direct and indirect activities of  $IFN-\gamma$  could suppress spontaneous tumor formation. Although  $IFN-\gamma$  can suppress tumor angiogenesis, it is unlikely that this is the main mode of action in preventing the development of nonsolid tumors such as disseminated lymphomas. It also remains feasible that loss of  $IFN-\gamma$  may result in tumor promotion by another factor normally suppressed by  $IFN-\gamma$ .

Gene-targeted mice have been extremely valuable when used in models where tumors are induced by viruses or carcinogens or simply when the mice were aged for spontaneous tumor formation. The value of using several types of tumor induction models is no better illustrated by the lack of tumor development in  $TNF^{-/-}$  mice, but the reduced predisposition of these mice to carcinogen-induced skin tumors (38). Although some other recent studies in aging mice have suggested that tumor immunosurveillance may exist (16, 36), our study directly demonstrated the specific importance of  $IFN-\gamma$  in the development of lymphomas and adenocarcinomas. Recently, a study of aging 129/Sv mice doubly deficient for recombination activating gene ( $RAG$ )-2 and signal transducer and activator of transcription ( $STAT$ )-1, the transcription factor mediating signaling by  $IFN-\gamma$  and  $IFN-\alpha/\beta$  receptors ( $RAG-2^{-/-}STAT-1^{-/-}$ ), suggested that lymphocytes and  $IFN-\gamma$  may play an important role in host protection from breast and colon carcinomas (36). Mice deficient in  $STAT-1$  alone displayed a low incidence of mammary carcinoma, however  $STAT-1$  regulates far more genes than  $IFNs$  alone, including the mammary tumor suppressor,  $BRCA-1$  (39). The lack of lymphoma reported in 129/Sv  $STAT-1$  mice (32) may be because 129/Sv behave like the BALB/c strain. Nevertheless, we did not observe colon or breast adenocarcinomas in either BALB/c or C57BL/6 strains of  $IFN-\gamma$ -deficient mice. The development of mammary and colon adenocarcinomas in 129/SvEv  $RAG-2^{-/-}STAT-1^{-/-}$  mice may be a consequence of intestinal hyperplasia peculiar to 129  $RAG^{-/-}$  mice. By contrast, the lung adenocarcinomas observed in BALB/c  $pfp^{-/-}$  and BALB/c  $IFN-\gamma^{-/-}$  mice occurred in strains of mice with no evidence of predisposing epithelial hyperplasia. The concept of whether lymphocytes play a key role in immunosurveillance beyond their own regulation (i.e., of nonlymphoid tissues) has been debated for years (40). The adenocarcinomas observed in mice in our study suggested that future studies, monitoring spontaneous tumor development in gene-targeted mice alone and bred with other strains genetically prone to epithelial malignancies, will be most informative.

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