Deficiencies of GM–CSF and Interferon γ Link Inflammation and Cancer

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Abstract

Chronic inflammation contributes to carcinogenesis, but the underlying mechanisms are poorly understood. We report that aged granulocyte-macrophage colony stimulating factor (GM-CSF)-deficient mice develop a systemic lupus erythematosis (SLE)-like disorder associated with the impaired phagocytosis of apoptotic cells. Concurrent deficiency of interferon (IFN)-γ attenuates the SLE, but promotes the formation of diverse hematologic and solid neoplasms within a background of persistent infection and inflammation. Whereas activated B cells show a resistance to fas-induced apoptosis, antimicrobial therapy prevents lymphomagenesis and solid tumor development. These findings demonstrate that the interplay of infectious agents with cytokine-mediated regulation of immune homeostasis is a critical determinant of cancer susceptibility.

Key words: GM-CSF • IFN-γ • cancer • inflammation • SLE

Introduction

There is compelling evidence that chronic inflammation increases the risk of neoplasia (1). Diverse autoimmune diseases predispose to B cell lymphomas and visceral malignancies (2). Persistent infections with *Helicobacter pylori* or hepatitis B and C virus contribute to cancers of the stomach and liver (3). Inflammatory syndromes of the intestinal or bronchial epithelia promote carcinomas of the colon and lung (4). In these pathologies, unresolved inflammation provokes cell turnover, which together with carcinogen- or phagocyte-induced DNA damage, eventually culminates in transformation (5). The genetic factors that control the progression from chronic inflammation to cancer, however, are poorly defined.

As vaccination with irradiated tumor cells engineered to secrete GM-CSF or, to a lesser extent IL-3, stimulates tumor destruction (6), we hypothesized that the endogenous production of these cytokines function in tumor suppression. Previous work revealed that GM-CSF– or GM-CSF/IL-3–deficient mice manifest a lung pathology resembling pulmonary alveolar proteinosis and specific immune defects that confer an increased susceptibility to pathogen challenge

(7–9). Here we show that deficiencies of GM-CSF and IFN-γ modulate the spontaneous development of inflammation, infection, and cancer.

Materials and Methods

Mice. GM-CSF– (7), GM-CSF/IL-3– (9), and IFN-γ– (10) deficient mice were backcrossed at least nine generations onto the C57Bl/6 strain and housed under specific pathogen-free conditions. Homozygous double and triple deficient mice were obtained by intercrossing, and the genotypes were confirmed by PCR. One cohort of GM-CSF/IL-3/IFN-γ–deficient mice was maintained from birth on enrofloxacin (Baytril®; Bayer) dissolved in chlorinated drinking water (85 mg/liter corresponding to a dose of 50 mg/kg). All mouse experiments were approved by the AAALAC-accredited Dana-Farber Cancer Institute IACUC.

Pathology. Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin. Immunohistochemistry was performed using standard techniques with monoclonal antibodies to CD4, CD8, B220, and Ig-κ (BD Biosciences).

ELISAs. Anti-double stranded DNA antibodies were measured by adding sera (diluted 1:100 in PBS) to 96 well ELISA plates (Nunc) coated with S1 nuclease-treated calf thymus DNA as described (11). After washing, the plates were incubated with an alkaline phosphatase-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) and developed with p-nitrophenyl phosphate (Boehringer). The absorbances at 405 nm were determined. Anti-C1q reactivity was measured by adding sera

(diluted 1:50 in PBS/1M NaCl) to an ELISA plate coated with human C1q (Quidel Corp.) as described (12). After washing, the plates were incubated with an alkaline phosphatase–conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) and developed as above. Serum immunoglobulins were quantified with an ELISA using antibodies from Southern Biotechnology Associates, Inc. and BD Biosciences according to the manufacturer's recommendations. B cell supernatants were concentrated with Microcon YM-3 filters (Millipore) and analyzed in a sandwich ELISA with anti–TNF-α or anti–IFN-γ monoclonal antibodies (BD Biosciences). The plates were developed with streptavidin-linked europium (Wallac Oy), and the fluorescence was measured with a Wallac Victor^2 1420 multilabel counter.

Phagocytosis of Apoptotic Cell. Thymocytes from 3–5-wk-old GM-CSF–deficient mice were exposed to 1 μM dexamethasone in RPMI plus 10% fetal calf serum for 6 h to induce a 60% annexin V positive and 95% propidium iodide negative population. 10^7 treated thymocytes were injected into the peritoneal cavities of 3–4-mo-old wild-type, GM-CSF–, or GM-CSF/IL-3–deficient mice. 30 min later the animals were killed and the peritoneal cavities lavaged with 3 ml of ice-cold PBS/0.3% BSA/0.03% EDTA/0.15% NaN$_3$. Cytospins were stained with Diff-Quik (Dade Behring AG). FACS® analysis of phagocytosis was performed by labeling the treated thymocytes before injection with 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester (5[6]-TAMRA, SE; Molecular Probes) as described (13). Macrophages were detected with FITC-conjugated-Mac-1 (BD Biosciences).

Lymphomas. Tumors were excised, processed to single cells, and cryopreserved. Lymphoma-derived DNA was digested with HindIII or EcoR1 and analyzed by southern using a J$_κ$ or J$_H$ probe, respectively, as described (14, 15). Single cell lymphoma suspensions were stimulated with antibodies to CD40 (BD Biosciences), and spectral karyotyping of metaphase spreads was performed as described (15).

Apoptosis Assays. Enriched B cells (90% pure) were obtained from spleens using negative selection with antibodies against CD4, CD8, and Mac-1 (Cedarlane). 5×10^6 cells were stimulated for 48 h with 1 μg of anti-CD40 antibody or LPS (50 μg) in 1 ml of RPMI and 10% fetal calf serum (resulting cultures were >90% B cells). 10^6 cell aliquots were then incubated with 1 μg of anti-fas antibody (Jo2; BD Biosciences), 100 μM etoposide (Sigma-Aldrich), 1 μM staurosporine (Sigma-Aldrich), or 2 μg/ml of actinomycin D (Sigma-Aldrich) for 12 h and cell viability determined by trypan blue exclusion. Anti–mouse TNF-α, isotype control antibodies (1 μg), and recombinant cytokines were from BD Biosciences.

Online Supplemental Material. The supplemental figures show clonal Ig heavy chain gene rearrangements (Fig. S1 A) and spectral karyotype (Fig. S1 B) of six B cell lymphomas. The supplemental tables detail tumor types (Table S1) and selected pathologies (Table S2) in GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20021258/DC1.
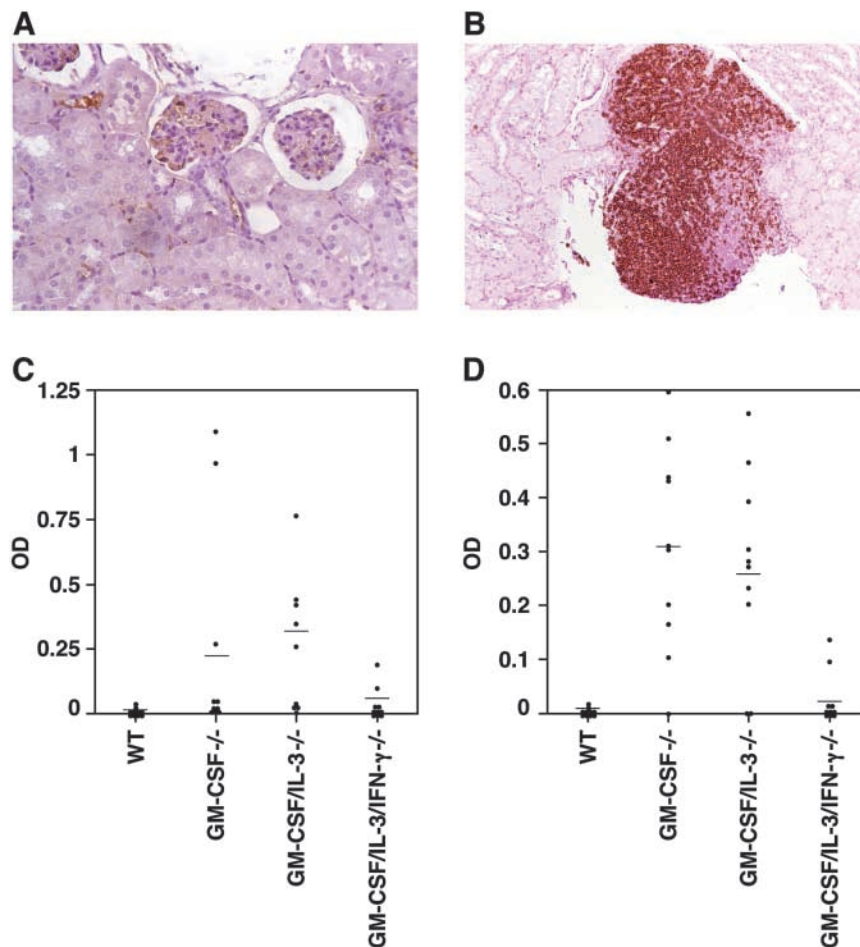

Figure 1. Aged GM-CSF– and GM-CSF/IL-3–deficient mice develop a SLE-like disorder. (A) Membrano-proliferative glomerulonephritis with Ig deposition in a GM-CSF-deficient mouse, anti-κ antibody, original magnification ×400. (B) B220$^+$ B cell aggregates in the renal pelvis of a GM-CSF–deficient mouse, original magnification ×400. (C) Anti-double stranded DNA antibodies. (D) Anti-C1q reactivity.

Results

GM-CSF–deficient Mice Develop SLE. While earlier work delineated a pulmonary alveolar proteinosis-like disease in GM-CSF– and GM-CSF/IL-3–deficient mice (7, 9), pathologic analysis of aged mutant animals (8+ months) on the C57Bl/6, but not Balb/c background, unexpectedly revealed immune-mediated glomerulonephritis with B and T cell aggregates in the renal pelvis and liver (Fig. 1, A and B; similar findings in 22/22 GM-CSF– and 17/17 GM-CSF/IL-3–deficient mice). Consistent with these lesions, a significant proportion of GM-CSF–and GM-CSF/IL-3–deficient mice generated anti-double-stranded DNA antibodies and anti-C1q-reactivity (Fig. 1, C and D). Together, these pathologic and serologic features characterize a SLE-like disorder in older mutant animals.

The mechanisms underlying SLE in several murine models involve the impaired phagocytosis of apoptotic cells (16). To test whether GM-CSF/IL-3 deficiency results in a similar defect, we injected dexamethasone-induced apoptotic thymocytes into peritoneal cavities and measured their phagocytosis by resident macrophages (13). Cytospins of peritoneal washings disclosed a marked reduction in the uptake of apoptotic bodies by macrophages from mutant mice compared with wild-type animals (Fig. 2, A and B). Similar defects were also present in GM-CSF deficient mice (5/5 animals examined, unpublished data). Moreover, fluorescent labeling of thymocytes with 5- (and 6-) carboxytetramethyl-rhodamine, succinimidyl ester (5[6]-TAMRA, SE) revealed that fewer than one-third of the Mac-1–positive cells from GM-CSF/IL-3–deficient mice were associated with apoptotic material, in contrast to more than three-quarters of the

Mac-1 positive cells from control animals (Fig. 2, C and D). This compromised phagocytosis of apoptotic cells is consistent with prior work showing the defective uptake of pulmonary surfactant, bacteria, and latex beads by alveolar macrophages from mutant mice (17). Whereas all mutant mice analyzed showed the impaired phagocytosis of apoptotic cells, the variable titers of anti-dsDNA antibodies indicate that additional factors contribute to disease severity.

GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient Mice. Despite the autoimmune disease, GM-CSF– and GM-CSF/IL-3-deficient mice failed to manifest an increase in spontaneous tumor formation over 14 mo of observation. As IFN-γ–deficient mice display an enhanced susceptibility to chemical carcinogenesis (18–20), we tested whether these cytokines cooperate in tumor suppression by generating compound knockout animals. Surprisingly, GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice demonstrated reduced survival compared with parental strains (Fig. 3 A). Whereas the litter sizes were nearly normal, ∼40% of the newborn animals succumbed to overwhelming pneumonia during the first few weeks of life (Fig. 3 C). Lung washings consistently grew *Pasteurella pneumotropica*, a gram-negative coccobacillus that normally colonizes the oropharynx of rodents without causing disease (21). Lethal infection in the mutant animals likely reflects, at least in part, diminished bacterial ingestion and killing.

Surviving 2–4-mo-old GM-CSF/IFN-γ–deficient mice manifested no alterations in the numbers of circulating blood cells, but GM-CSF/IL-3/IFN-γ–deficient animals showed a mild eosinophilia ($0.29 \pm 0.12 \times 10^3$, GM-CSF/IL-3/IFN-
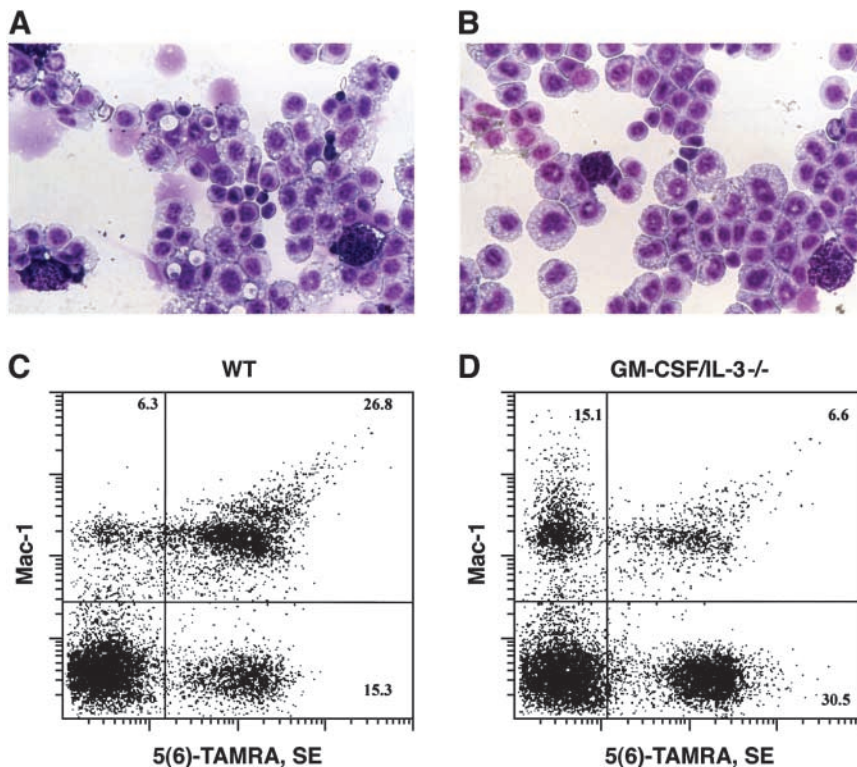

Figure 2. Impaired phagocytosis of apoptotic cells in GM-CSF– and GM-CSF/IL-3–deficient mice. Dexamethasone-induced apoptotic thymocytes from GM-CSF–deficient mice were injected into the peritoneal cavities of wild-type or GM-CSF/IL-3–deficient mice. Stained cytospins of harvested cells from: (A) wild-type mouse, original magnification ×400. (B) GM-CSF/IL-3–deficient mouse, original magnification ×400. Similar results were obtained in five experiments, and GM-CSF-deficient mice manifested comparable defects. (C and D) Apoptotic thymocytes were labeled with 5[6]-TAMRA before injection, and FACS® was used to analyze the harvested peritoneal cells. (C) Wild-type mouse. (D) GM-CSF/IL-3–deficient mouse. Similar results were found in six experiments.
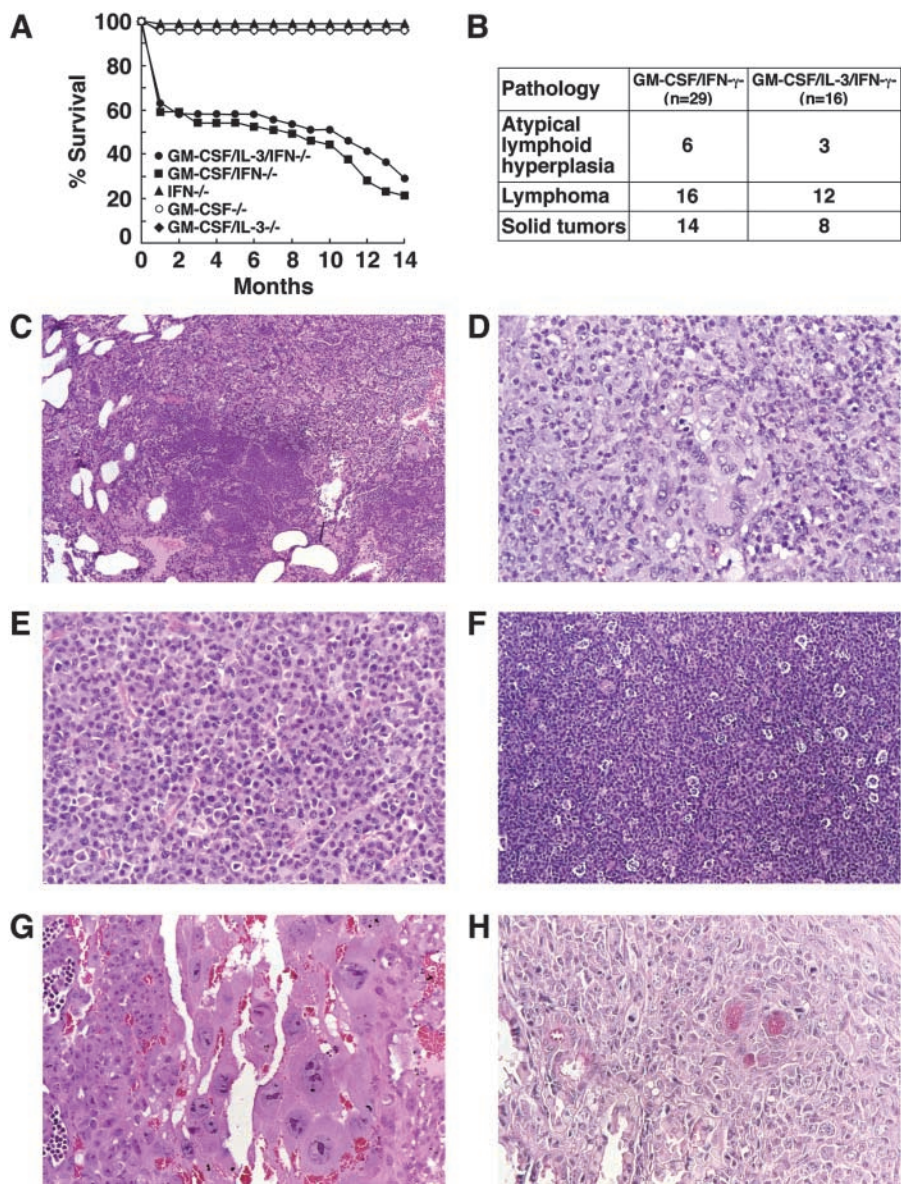
Figure 3. Infection, inflammation, and cancer in GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice. (A) Survival curves for mice singly or multiply deficient in GM-CSF, IL-3, and IFN-γ. The numbers of animals per cohort were: GM-CSF/IL-3/IFN-γ– (*n* = 29), GM-CSF/IFN-γ– (*n* = 44), IFN-γ– (*n* = 14), GM-CSF– (*n* = 18), GM-CSF/IL-3– (*n* = 18). (B) Spectrum and incidence of spontaneous tumors. The number of mice harboring tumors at autopsy is shown. (C) Lethal bacterial pneumonia, H&E stain, original magnification ×400. (D) Granulomatous inflammation with multinucleated giant cells, H&E stain, original magnification ×400. (E) Plasmacytoid lymphoma, H&E stain, original magnification ×400. (F) High grade large cell lymphoma, H&E stain, original magnification ×400. (G) Choriocarcinoma, H&E stain, original magnification ×200. (H) Mucin positive biliary tract/liver carcinoma, mucicarmine stain, original magnification ×400.

γ–deficient versus $0.08 \pm 0.03 \times 10^3$, wild-type; P = 0.0001) that was comparable to GM-CSF/IL-3–deficient mice ($0.32 \pm 0.10 \times 10^3$; reference 9). The thymi of GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient animals maintained normal T cell maturation, and the spleens contained normal numbers of B220, CD3, CD4, CD8, NK1.1, NK1.1-T, and CD11c-positive cells, although Gr-1 and Mac-1 positive cells were increased twofold.

Over time GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice gradually became moribund, with most requiring sacrifice by 14 mo. At autopsy, acute and chronic inflammatory reactions were present in many organs, particularly the lungs, soft tissues, lymph nodes, ovaries, adrenal glands, and liver (Fig. 3 D). *Pasteurella pneumotropica* and enterococcal species were frequently cultured from these lesions. Bone marrow myeloid hyperplasia and splenic extra-medullary hematopoiesis were associated with the per-

sistent infections. Inflammatory lesions were present in 39/45 mice examined.

In contrast to the intense inflammatory responses, the SLE-like disorder was attenuated in GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice compared with GM-CSF– and GM-CSF/IL-3–deficient animals. Lymphoid aggregates were absent from the renal pelvis, and the immune-mediated glomerulonephritis was less severe. Moreover, anti-double-stranded DNA antibodies and anti-C1q reactivity were reduced (Fig. 1, C and D). Together, these results reveal a critical role for IFN-γ in the SLE induced by GM-CSF deficiency and are in accordance with the requirement for IFN-γ in the SLE caused by defective fas/fas-ligand function (22).

Spontaneous Tumors. Within the background of chronic infection and inflammation, a high proportion of GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice devel-

oped lymphoproliferative diseases (Fig. 3 B, online supplemental Table S1). Atypical lymphoid hyperplasias (23) originating in mesenteric lymph nodes or Peyer's patches frequently evolved to mature B cell lymphomas (B220[+], sIg[+] by immunohistochemistry) involving the liver, spleen, and other organs (36/40 lymphomas examined were B cell). Pathologically, the tumors ranged from low-grade lesions with plasmacytoid features to high-grade large cell expansions with abundant mitoses (Fig. 3, E and F). Southern analysis of immunoglobulin light and heavy chain gene rearrangements established that the proliferations were clonal (Fig. 4 A, and online supplemental Fig. S1 A). Spectral karyotyping further disclosed chromosomal translocations and aneuploidy in 3 of 6 tumors examined (Fig. 4 B, and online Supplemental Fig. S1 B). Three of three lymphomas tested were efficiently transplanted into young GM-CSF/IL-3/IFN-γ–deficient recipients (unpublished data).

In addition to the lymphomas, nearly 50% of the GM-CSF/IFN-γ– and GM-CSF/IL-3/IFN-γ–deficient mice developed solid tumors (Fig. 3 B, online supplemental Table S1, online supplemental Table S2). The pathologies observed ranged from lesions that resemble well-described benign tumors in the human to carcinomas with metastases. Ovarian tumors were the most common, with 6/13 double deficient and 4/4 triple deficient females manifesting choriocarcinomas (Fig. 3 G), luteomas, or teratomas. The broad spectrum of tumors also included carcinomas of the biliary tract/liver (Fig. 3 H), salivary gland, and bladder as well as adenomas/hyperplasias of the pancreatic islets, seminal vesicle, and osteochondral junction. It is

noteworthy that 4/29 double deficient and 4/16 triple deficient mice showed multiple solid lesions.

Tumor development required the combined deficiency of GM-CSF and IFN-γ. Histopathologic analysis of aged animals (12–17 mo) revealed no tumors in 7 wild-type mice, 2 cases of atypical hyperplasia and 1 luteoma in 15 GM-CSF-deficient mice, 2 cases of atypical hyperplasia in 17 GM-CSF/IL-3–deficient mice, and 1 luteoma and 1 islet cell hyperplasia in 13 IFN-γ–deficient mice. Moreover, gross autopsy of 28 additional IFN-γ–deficient animals up to 27 mo of age disclosed only a single case of lymphoma (B220[+]). Most GM-CSF- and GM-CSF/IL-3–deficient mice succumbed to pulmonary alveolar proteinosis by 18 mo, precluding a longer-term study of spontaneous tumor development in these strains.

Lymphomagenesis. To explore the mechanisms underlying spontaneous tumor formation in the compound cytokine deficient mice, we characterized potential precursor lesions in B cell lymphomagenesis. Although secondary lymphoid tissues of 2–3 mo old mice did not show evidence of atypical hyperplasia or clonal proliferation, germinal centers were enlarged. Consistent with these findings, serum IgG1 levels were higher in mutant mice than controls (1,811 ± 329, GM-CSF/IFN-γ–deficient versus 921 ± 172, wild-type; P = 0.0003); serum IgG2b (4,431 ± 2,150 versus 2,240 ± 730) and IgE levels (845 ± 709 versus 330 ± 427) were also increased, but these did not reach statistical significance.

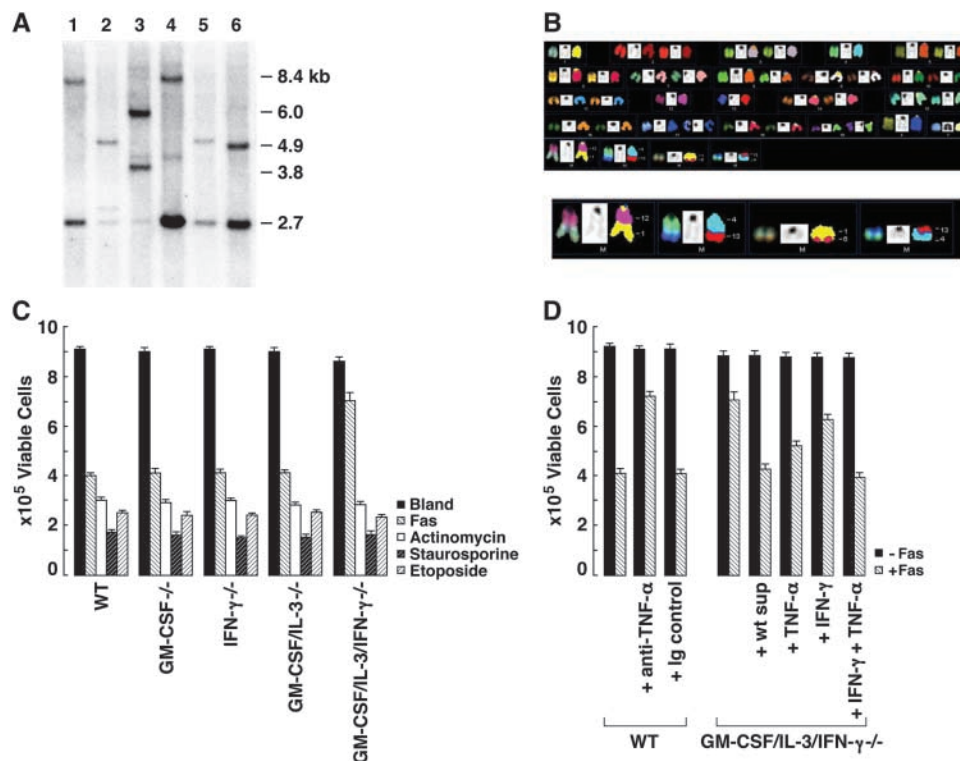Unresolved infection and inflammation likely contribute to the persistent B cell activation in GM-CSF/IFN-γ–

Figure 4. B cell lymphomas. (A) Tumor-derived DNA from six different lymphomas was analyzed by southern using a J_κ probe. The germline band is 2.7 kb. (B) Single cell lymphoma suspensions were stimulated with anti-CD40 antibodies, and spectral karyotyping of metaphase spreads was performed. Shown are clonal 12:1, 4:13, 13:4, and 1:6 chromosomal translocations in one case. Chromosomal abnormalities were found in 3 of 6 tumors. (C) Impaired fas-mediated apoptosis. Enriched splenic B cells (90% pure) were stimulated for 48 h with anti-CD40 antibody (after activation, >90% B cells), and then 10[6] cell aliquots were incubated with 1 μg of anti-fas antibody, 100 μM etoposide, 1 μM staurosporine, or 2 μg/ml of actinomycin D for 12 h and cell viability determined. Data are pooled from six experiments; fas-treated wild-type versus fas-treated GM-CSF/IL-3/IFN-γ deficient, P < 0.0001. Similar results were obtained with LPS (50 μg) stimulated B cells. A comparable fas-resistance was also found with GM-CSF/IFN-γ–deficient B cells. (D) TNF-α and IFN-γ are required for fas-mediated B cell apoptosis. The data are pooled from four experiments.

and GM-CSF/IL-3/IFN-γ–deficient mice. To determine whether intrinsic B cell defects were also involved, we evaluated the responses of purified B cells to anti-CD40 antibodies or lipopolysaccharide. No significant differences in the cell cycle profiles or up-regulation of surface MHC II, B7–1, and fas expression were detected between the mutant and wild type cells after 48 h of stimulation (unpublished data). Nonetheless, activated B cells from GM-CSF/IL-3/IFN-γ–deficient mice manifested a resistance to fas-mediated apoptosis ($P < 0.0001$, versus wild-type), whereas activated B cells from IFN-γ–, GM-CSF–, or GM-CSF/IL-3–deficient animals displayed fas sensitivities equivalent to controls (Fig. 4 C). The killing induced with etoposide, staurosporine, and actinomycin, in contrast, was comparable among all the strains.

As soluble factors modulate target cell fas sensitivity (24), we tested whether conditioned media from activated wild-type B cells could reverse the defective response of GM-CSF/IL-3/IFN-γ–deficient B cells. As illustrated in Fig. 4 D, the addition of wild-type supernatants to mutant cultures restored the fas sensitivity, suggesting that the impaired function of one or more cytokines might be responsible for the defect. Indeed, anti–TNF-α antibodies significantly inhibited fas killing in wild-type B cells, and supernatants from wild-type cultures contained fivefold more immunoreactive TNF-α (6.7 ± 0.44 pg/5 × 10^6 cells) than GM-CSF/IL-3/IFN-γ–deficient cultures (1.3 ± 0.21 pg/5 × 10^6 cells), as measured by ELISA. The restoration of normal TNF-α levels in the mutant cultures with recombinant cytokine partially corrected the fas resistance (Fig. 4 D). However, the restoration of both wild-type TNF-α and IFN-γ levels (2.5 ± 0.06 pg/5 × 10^6 cells) completely restored the fas sensitivity of mutant cells. These results establish a dual requirement for TNF-α and IFN-γ in fas-mediated B cell apoptosis. As aged fas-deficient mice harbor B cell lymphomas (25), the apoptotic defect delineated here may contribute to tumor development. However, further studies are required to determine whether a similar TNF-α deficiency is present in vivo.

Antibiotics Suppress Tumor Formation. As microbial agents contribute to lymphomagenesis and solid tumor development in humans, we maintained a cohort of 24 GM-CSF/IL-3/IFN-γ-deficient mice from birth on enrofloxacin. Consistent with the ability of this antibiotic to suppress *Pasteurella pneumotropica* and enterococcal species (21), early infectious deaths were eliminated from the cohort. Moreover, antimicrobial therapy prevented tumor formation in aged animals. No lymphomas or solid tumors were detected in 19 mice autopsied at 14 mo ($P < 0.001$ for lymphomas, $P < 0.001$ for all solid tumors, and $P = 0.03$ for carcinomas, by the Fisher exact test, compared with the untreated cohort). One animal showed atypical thymic hyperplasia; 3 mice died of fighting-related injuries and 2 of unknown causes between 8 and 12 mo. The suppression of tumor formation was associated with a marked reduction in chronic inflammatory lesions; of 9 animals studied by histopathology, only 1 showed mild cholangitis and pancreatitis. In contrast, CD40-activated B cells from enrofloxacin-treated

mice displayed a comparable fas-resistance to lymphocytes from untreated GM-CSF/IL-3/IFN-γ–deficient animals (2 mice analyzed, unpublished data). Additional experiments are required to determine whether anti-microbial therapy will suppress tumor formation over a longer time period.

Discussion

Our studies establish that the interplay of GM-CSF and IFN-γ deficiencies modulates susceptibility to inflammation, infection, and cancer. In this context, recent experiments in *C. elegans* demonstrate that a partial impairment of caspase activity cooperates with an engulfment defect to allow the recovery of some cells otherwise committed to apoptosis (26, 27). We propose that in the germinal center reactions of GM-CSF/IFN-γ–deficient mice, B cell survival is similarly enhanced by a combination of the fas resistance and the impaired phagocytosis of early apoptotic cells (28). In the setting of chronic antigenic stimulation, this expanded pool of B cells acquires an increased risk for transforming mutations due to rare errors in somatic hypermutation (29).

Our finding that antimicrobial therapy inhibits both lymphomagenesis and solid tumor formation is consistent with clinical observations that a variety of infectious agents are linked with some human B cell lymphomas and solid neoplasms, including carcinomas of the stomach, liver, biliary tract, and bladder (3). The model reported here should prove useful for clarifying whether microbes function as cofactors that drive target cell proliferation and/or directly mediate target cell transformation. The antibiotic-mediated suppression of inflammatory pathology also supports the idea that tissue damage secondary to unresolved inflammation is an important contributor to carcinogenesis (5). Together, these results indicate that the complex interaction of infectious agents and defective immune homeostasis is a critical determinant of cancer susceptibility.

Our studies further suggest that the high incidence of T cell lymphomas recently reported in IFN-γ single deficient mice on the C57Bl/6 background (30) reflects, at least in part, the involvement of an infectious agent not present in our colony. Interestingly, spontaneous lymphomas were not increased in IFN-γ single deficient mice on the Balb/c background, although IFN-γ ablation in this strain enhanced the B cell lymphomagenesis consequent to perforin deficiency (30); these latter results are consistent with the findings presented here.

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