B regulatory cells and the tumor-promoting actions of TNF-α during squamous carcinogenesis

Tiziana Schioppa[a], Robert Moore[a], Richard G. Thompson[a], Elizabeth C. Rosser[b], Hagen Kulbe[a], Sergei Nedospasov[c,d], Claudia Mauri[b], Lisa M. Coussens[e,f], and Frances R. Balkwill[a,1]

[a]Centre for Cancer and Inflammation, Barts Cancer Institute, Queen Mary University of London, London EC1M 6BQ, United Kingdom; [b]Centre for Rheumatology Research, Department of Medicine, University College London, London W1 4JF, United Kingdom; [c]Engelhardt Institute of Molecular Biology, Moscow 119991, Russia; [d]German Rheumatism Research Center, a Leibniz Institute, D-13553 Berlin, Germany; and [e]Department of Pathology and [f]Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA 94143

Edited by Michael Karin, University of California, San Diego School of Medicine, La Jolla, CA, and approved May 23, 2011 (received for review January 19, 2011)

The inflammatory cytokine TNF-α has been recognized as a critical tumor promoter, but the effector cells that mediate its action have not been fully characterized. Because B cells regulate squamous and prostate carcinogenesis, and *Tnf*$^{-/-}$ mice harbor B-cell defects, we investigated the hypothesis that B cells are important effector cells for TNF-α–mediated promotion of cancer development. Using an adoptive transfer strategy and the 7,12-dimethylbenz[α]anthracene/terephthalic acid (DMBA/TPA) two-stage model of skin carcinogenesis, we found that both B cells and TNF-α are critical for the development of DMBA/TPA-induced papilloma. Transfer of B cells from DMBA/TPA-treated wild-type mice to *Tnf*$^{-/-}$ mice rescued papilloma development to a wild-type level, a result not observed when B cells from *Tnf*$^{-/-}$ mice were transferred to *Rag2*$^{-/-}$ mice or when TNF-α was eliminated selectively in B cells. Resistance to papilloma development in *Tnf*$^{-/-}$ mice was associated with increased IFN-γ and CD8^{+} T cells in skin and a significant reduction in IL-10–producing B regulatory cells alongside an increase in IFN-γ–producing CD8^{+} T cells in the spleen. These data indicate that during DMBA/TPA-induced squamous carcinogenesis TNF-α mediates tumor-promoting activity via regulatory B cells that repress antitumor immunity.

immune cells | inflammation | tumor microenvironment

In 1999, we reported that mice deficient in the cytokine TNF-α are resistant to chemical carcinogenesis of the skin (1). Despite the name and history of this cytokine, we concluded that TNF-α is an endogenous tumor promoter in the mouse 7,12-dimethylbenz[α] anthracene/12-O-tetradecanoylphorbol 13-acetate (DMBA/TPA) model of skin carcinogenesis. It now is clear that TNF-α is involved in promotion and progression in a range of genetic, chemically induced, and transplantable (syngeneic and xenograft) mouse models of cancer (e.g., refs. 2–6).

These findings are supported by the presence of TNF-α in biopsies of human cancer, elevated plasma levels of TNF-α in some cancer patients, and by phase I/II clinical trials of TNF-α antagonists (6). The protumor actions of this inflammatory cytokine are mediated primarily via p55 TNF receptor 1 (7, 8) with evidence for autocrine actions on malignant cells (5) as well as paracrine actions on myeloid cells (9, 10) and CD4^{+} T cells (11).

B cells have a critical tumor-promoting role in a genetic model of squamous carcinoma (12), mediated by humoral immunity and Fc receptors expressed on infiltrating myeloid cells (13). Because a there is a well-documented deficiency in B-cell responses in *Tnf*$^{-/-}$ and *Tnfr1*$^{-/-}$ mice (14–17), we hypothesized that defects in B-cell responses might contribute to the resistance of *Tnf*$^{-/-}$ mice to skin carcinogenesis and that TNF-α might be involved in the tumor-promoting actions of B cells.

Results and Discussion

We conducted experiments in immune-deficient and *Tnf*$^{-/-}$ mice using the two-stage carcinogenesis model to induce skin inflammation and papillomas. A single dose of the carcinogen DMBA was administered topically to the shaved dorsal skin of 6- to 8-wk-old mice (the hair growth arrest phase), and tumor development was promoted by repeated administration of TPA for 15 wk.

B cell- and T cell-deficient C57BL/6 *Rag2*$^{-/-}$ mice were resistant to papilloma development compared with wild-type C57BL/6 mice (Fig. 1*A*). This result indicated that B and/or T cells, or their mediators, are necessary for tumor promotion, as reported for skin tumor development in K14-HPV16 transgenic mice (12, 13). Because B cell–deficient *Jh* mice were less susceptible to papilloma development ($P < 0.05$; Fig. 1*B*), B cells became our primary focus. Wild-type and *Rag2*$^{-/-}$ mice were treated with DMBA and then with TPA for 4 wk; after treatment for 4 wk, epidermal dysplasia was evident in all wild-type mice, but papillomas had not yet emerged. *Rag2*$^{-/-}$ mice then were reconstituted with wild-type splenic CD19^{+} B cells from mice that had undergone the same DMBA/TPA treatment, and promotion with TPA continued for 11 wk. This treatment significantly restored susceptibility to papilloma development ($P < 0.05$) in *Rag2*$^{-/-}$ mice (Fig. 1*C*), supporting a role for B cells as mediators of skin tumor promotion.

To determine if B-cell defects in *Tnf*$^{-/-}$ mice contributed to their resistance to skin carcinogenesis, splenic CD19^{+} B cells isolated from DMBA/TPA-treated C57BL/6 wild-type mice were adoptively transferred to DMBA/TPA-treated C57BL/6 *Tnf*$^{-/-}$ mice as above. Transfer of splenic B cells from DMBA/TPA-treated wild-type mice significantly increased papilloma development in *Tnf*$^{-/-}$ mice (Fig. 1*D*) ($P < 0.05$). In contrast, B cells from DMBA/TPA-treated *Tnf*$^{-/-}$ mice failed to restore papilloma development in DMBA/TPA-treated *Rag2*$^{-/-}$ mice (Fig. 1*E*). We then used mice in which TNF-α was deleted in B cells (CD19-Cre × *Tnf*$^{f/f}$ mice) (Fig. 1*F* and Fig. S1) (17). A selective lack of TNF-α in B cells significantly reduced papilloma development compared with wild-type mice ($P < 0.002$) (Fig. 1*F*) as well as with control CD19-Cre or *Tnf*$^{f/f}$ groups (both $P < 0.05$). These data demonstrate that B cells producing TNF-α are important in papilloma development.

To understand better the tumor-promoting roles of B cells and TNF-α in skin carcinogenesis, we compared skin leukocyte infiltration (CD45^{+}, Gr-1^{+}, CD8^{+}, and F4/80^{+} cells and mast cell serine esterase activity) and cytokine/chemokine levels in wild-type and *Tnf*$^{-/-}$ mice at 2, 7, and 10 wk of DMBA/TPA treatment. CD8^{+} T cells were present in significantly higher numbers in DMBA/TPA-treated *Tnf*$^{-/-}$ mouse skin than in the wild-type skin ($P < 0.05$) (Fig. 2*A*, *Left*). F4/80^{+} macrophages were sig-

Author contributions: F.R.B. designed research; T.S., R.M., R.G.T., and E.C.R. performed research; T.S., R.M., R.G.T., H.K., S.N., C.M., and L.M.C. contributed new reagents/analytic tools; T.S., R.M., and E.C.R. analyzed data; and T.S. and F.R.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: f.balkwill@qmul.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1100994108/-/DCSupplemental.

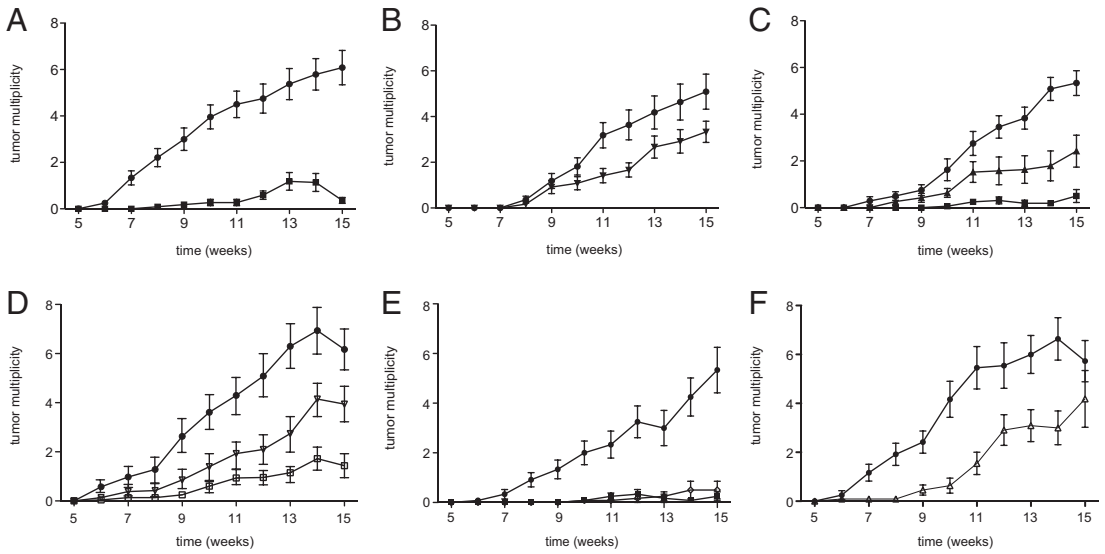

Fig. 1. B cells are involved in DMBA/TPA squamous carcinogenesis. (*A*) *Rag2*$^{-/-}$ mice are resistant to DMBA/TPA. ●, C57/Bl6 wild-type mice (n = 24); ■, C57/Bl6 *Rag2*$^{-/-}$ mice (n = 23); $P < 0.0001$. This graph shows combined data from two independent experiments. (*B*) Mice deficient for B cells (*Jh* mice) are partially resistant to DMBA/TPA skin carcinogenesis. ●, BALB/c wild-type mice (n = 12); ▼, BALB/c *Jh* mice (n = 12); $P < 0.05$. (*C*) Wild-type B cells partially rescue the resistance to carcinogenesis of *Rag2*$^{-/-}$ mice. ●, wild-type mice (n = 24); ■, *Rag2*$^{-/-}$ mice (n = 16); ▲, mice with *Rag2*$^{-/-}$ plus wild-type B cells (n = 21). Wild-type mice versus *Rag2*$^{-/-}$ mice, $P < 0.001$; mice with *Rag2*$^{-/-}$ plus wild-type B cells versus *Rag2*$^{-/-}$ mice, $P < 0.05$; mice with *Rag2*$^{-/-}$ plus wild-type B cells versus wild-type mice = no significant difference. This graph shows combined data from two independent experiments. (*D*) Wild-type B cells partially rescue the *Tnf*$^{-/-}$–resistant phenotype. ●, C57/Bl6 wild-type mice (n = 36); □, C57/Bl6 *Tnf*$^{-/-}$ mice (n = 2); ▽, C57/Bl6 mice with *Tnf*$^{-/-}$ plus wild-type B cells (n = 36). $P < 0.05$. Wild-type mice versus *Tnf*$^{-/-}$ mice, $P < 0.001$; mice with *Tnf*$^{-/-}$ plus wild-type B cells versus *Tnf*$^{-/-}$ mice, $P < 0.05$; mice with *Tnf*$^{-/-}$ plus wild-type B cells versus wild-type mice = no significant difference. This graph shows combined data from three independent experiments. (*E*) *Tnf*$^{-/-}$ B cells are not able to rescue the resistant phenotype of *Rag2*$^{-/-}$ mice. ●, C57/Bl6 wild-type mice (n = 12); ♢, mice with *Rag2*$^{-/-}$ plus *Tnf*$^{-/-}$B cells (n = 11); ■, C57/Bl6 *Rag2*$^{-/-}$ mice (n = 10); Wild-type mice versus *Rag2*$^{-/-}$ mice, $P < 0.001$; mice with *Rag2*$^{-/-}$ plus *Tnf*$^{-/-}$ B cells versus wild-type mice, $P < 0.001$; mice with *Rag2*$^{-/-}$ plus *Tnf*$^{-/-}$ B cells versus *Rag2*$^{-/-}$ mice = no significant difference. (*F*) CD19-*Cre*/*Tnf*$^{f/f}$ mice are partially resistant to DMBA/TPA. ●, C57/Bl6 wild-type mice (n = 12); △, C57/Bl6 CD19^{Cre}/*Tnf*$^{f/f}$ mice (n = 12); difference between the groups, $P < 0.002$. Error bars indicate SE.

nificantly decreased in *Tnf*$^{-/-}$ mice compared with wild-type mice at 2 and 7 wk ($P < 0.05$; Fig. 2*B*, *Left*). There were no other significant differences in leukocyte infiltrate.

CCL5, a T-cell chemoattractant, was increased threefold in untreated *Tnf*$^{-/-}$ epidermis compared with wild-type epidermis (Fig. 2*C*, *Left*) and also increased in DMBA/TPA-treated *Tnf*$^{-/-}$ epidermis compared with wild-type (Fig. 2*C*, *Center*). IFN-γ mRNA was 10-fold higher in untreated *Tnf*$^{-/-}$ epidermis than in untreated wild-type epidermis (Fig. 2*D*, *Left*) and was higher at all time points during DMBA/TPA treatment (Fig. 2*D*, *Center*). We found no significant differences in CXCL10, IL-10, IL-12p40, and p70 mRNA.

Adoptive transfer of B cells from DMBA/TPA-treated wild-type mice to DMBA/TPA-treated *Tnf*$^{-/-}$ mice significantly reduced the numbers of CD8$^+$ T cells in skin (Fig. 2*A*, *Right*; $P < 0.05$) and increased the numbers of F4/80$^+$ cells (Fig. 2*B*, *Right*). CCL5 mRNA was lower in *Tnf*$^{-/-}$ epidermis after transfer of wild-type B cells, as was IFN-γ mRNA at 10 wk (Fig. 2 *C* and *D*, *Right*).

Deposition of B lymphocyte-derived Ig and increased circulating immune complexes (CICs) in premalignant and malignant stroma are thought to be significant B-cell mediators of tumor promotion in human and murine carcinomas (13, 18, 19). Untreated wild-type and *Tnf*$^{-/-}$ mice had very low levels of C1q/IgG immune complexes that increased after DMBA/TPA treatment in both wild-type and *Tnf*$^{-/-}$ mice, but *Tnf*$^{-/-}$ mice exhibited reduced levels of C1q/IgG immune complexes at 2 ($P < 0.05$) and 7 wk ($P < 0.001$) compared with DMBA/TPA-treated wild-type mice. The immune complex levels were partially restored following transfer of wild-type splenic B cells isolated from DMBA/TPA-treated mice (Fig. 3*A*), but this difference was not sustained at the 10-wk time point, so we decided to look at other aspects of the immune response that can be influenced by B cells.

Because B cells also regulate immune responses via cytokine production, we isolated leukocytes from inguinal lymph nodes and measured cytokine production. The only significant difference between wild-type and *Tnf*$^{-/-}$ lymph node cells was in IL-10; in DMBA/TPA-treated mice, wild-type cells expressed eightfold higher levels than cells from *Tnf*$^{-/-}$ mice ($P < 0.001$; Fig. 3*B*). We then studied the ability of splenic B cells to produce cytokines in response to LPS and IgM, both strong B-lymphocyte activators (20). LPS-stimulated CD19$^+$ B cells isolated from *Tnf*$^{-/-}$ mice produced less IL-10 and more total IL-12 (IL-12p70) than did CD19$^+$ B cells from wild-type mice (Fig. 3*C*; $P < 0.05$).

Tnf$^{-/-}$ mice had a reduced absolute number of IL-10$^+$/CD19$^+$ B cells in their spleens (Fig. 4*A*; $P < 0.05$, Fig. S2*A*). CD19$^+$ B cells isolated from wild-type mice produced 10–25 pg IL-10 per 10^6 cells during short-term culture, but no IL-10 was found in B-cell supernatants from DMBA/TPA-treated *Tnf*$^{-/-}$ mice (Fig. 4*B*; $P < 0.05$).

Regulatory B cells produce IL-10 and exert immunosuppressive effects in models of autoimmunity (21). CD19$^+$ regulatory B cells have high levels of CD21 and are contained within the transitional two-marginal zone precursor subset (22). Our results suggested that *Tnf*$^{-/-}$ mice possessed fewer regulatory B cells, possibly contributing to their resistance to skin carcinogenesis. Splenic B cells from untreated and DMBA/TP- treated wild-type and *Tnf*$^{-/-}$ mice were analyzed by flow cytometry for differences in their expression of CD21. We found that *Tnf*$^{-/-}$ mice are deficient for CD19$^+$CD21^{high} B cells compared with wild-type mice, both in terms of percentage of cells and absolute number (Fig. 4*C*; $P < 0.01$, Fig. S2*B*).

Because we also found increased CD8$^+$ cells in *Tnf*$^{-/-}$ skin and more IFN-γ mRNA in epidermis, we analyzed IFN-γ positivity in CD3$^+$ T cells from spleens and inguinal lymph nodes. DMBA/TPA-treated *Tnf*$^{-/-}$ mice had more IFN-γ$^+$ T cells in spleens ($P < 0.05$) than wild-type treated mice (Fig. 4*D*). In addition

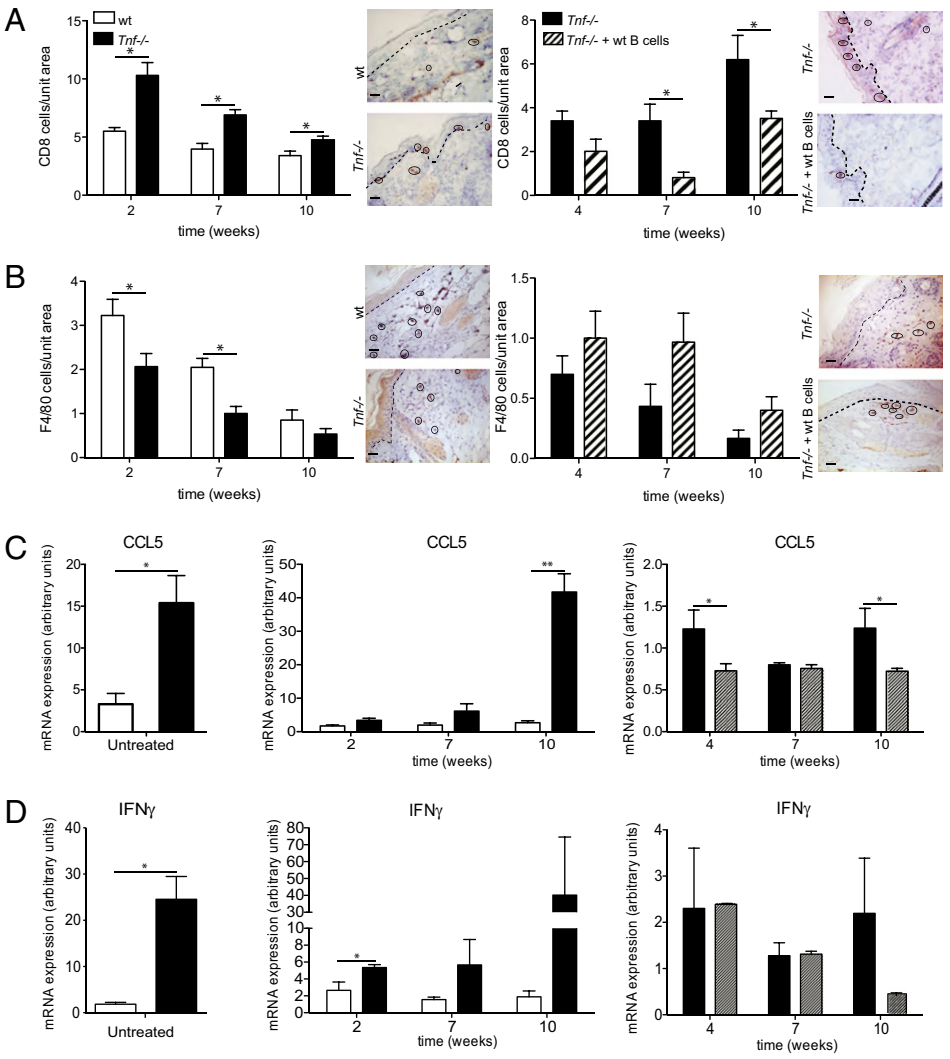

Fig. 2. Characterization of the inflammatory status of wild-type and $Tnf^{-/-}$ skin. (*A*) $CD8^+$ cells are more numerous in $Tnf^{-/-}$ mice than in wild-type mice (*Left*, wild-type, $n = 4$; $Tnf^{-/-}$, $n = 4$) and are reduced after transfer of wild-type B cells (*Right*, $Tnf^{-/-}$ $n = 3$; $Tnf^{-/-}$ plus wild-type B cells, $n = 3$). $^*P < 0.05$. (*B*) $F4/80^+$ cells are reduced in $Tnf^{-/-}$ mice compared with wild-type mice ($^*P < 0.05$) (*Left*, wild-type, $n = 4$; $Tnf^{-/-}$, $n = 4$) and are higher after transfer of wild-type B cells (*Right*, $Tnf^{-/-}$, $n = 3$; $Tnf^{-/-}$ plus wild-type B cells, $n = 3$). (*C*) CCL5 mRNA expression. Results are from pooled mRNA from several mice for each group: wild-type and $Tnf^{-/-}$, $n = 4$, $Tnf^{-/-}$, $n = 3$; and $Tnf^{-/-}$ plus wild-type B cells, $n = 3$. $^*P < 0.05$, $^{**}P < 0.001$. (*D*) IFN-γ mRNA expression. The results are from pooled mRNA from several mice for each group: wild-type and $Tnf^{-/-}$, $n = 4$; $Tnf^{-/-}$, $n = 3$; and $Tnf^{-/-}$ plus wild-type B cells, $n = 3$. $^*P < 0.05$. Error bars indicate SE.

a significantly increased presence of IFN-γ^+ T cells was found in inguinal lymph nodes from both untreated and DMBA/TPA-treated $Tnf^{-/-}$ mice compared with wild-type mice (Fig. 4*E*; $P < 0.05$). Therefore, resistance to papilloma development in $Tnf^{-/-}$ mice may result in part from increased $CD8^+$ IFN-γ–producing T cells that suppress development of premalignant lesions (Fig. S2*C*). We propose that this resistance is the result of reduced numbers of B regulatory cells that normally may restrain the antitumor activity of $CD8^+$ cells. This hypothesis is supported by experimental data shown in Fig. 4*F*. TNF-α blockade of LPS-induced B-cell activation significantly reduced IL-10 production (Fig. 4*F*; $P < 0.05$), with no difference in production of IL-4, -5, -2, or -12 or IFN-γ. In conclusion, we have demonstrated that B cells are a significant cellular source of TNF-α for the tumor-promoting actions of TPA. $Tnf^{-/-}$ mice have fewer IL-10–producing regulatory B cells, and our results suggest that their activity is regulated by TNF-α.

There has been speculation that B regulatory cells may promote tumor development (23, 24), and our results support this hypothesis in a mouse cancer model. B cells are important TNF-α producers, and TNF signaling is required for the development of follicular dendritic cells that initiate germinal center structures; here we demonstrate a link between B cells and the tumor-promoting actions of TNF-α. There is, however, increasing evidence of a role for B cells in the promotion of solid tumor development whereby immunoglobulins and CICs, acting via Fcγ receptors on myeloid cells, enhance protumor immunity, cancer-related chronic inflammation, and tumor development in a genetic model of squamous carcinogenesis (13, 18). Recent findings also have shown a role for B cells in shaping the protumoral function of macrophages (25), supporting the hypothesis that B cells might influence other immune cells in addition to T cells. There also is evidence that lymphotoxin production by tumor-infiltrating B cells promotes androgen-resistant prostate cancer (26). B cells also may negatively regulate immune responses to tumors (e.g., ref. 27), and in two models of transplantable cancer, antitumor responses were enhanced in B cell-deficient μ-MT mice via a mechanism involving increased IFN-γ and reduced IL-10 (28).

Although our experiments have highlighted the importance of B cells, the data from the $Rag2^{-/-}$ mice (Fig. 1*A*) compared with

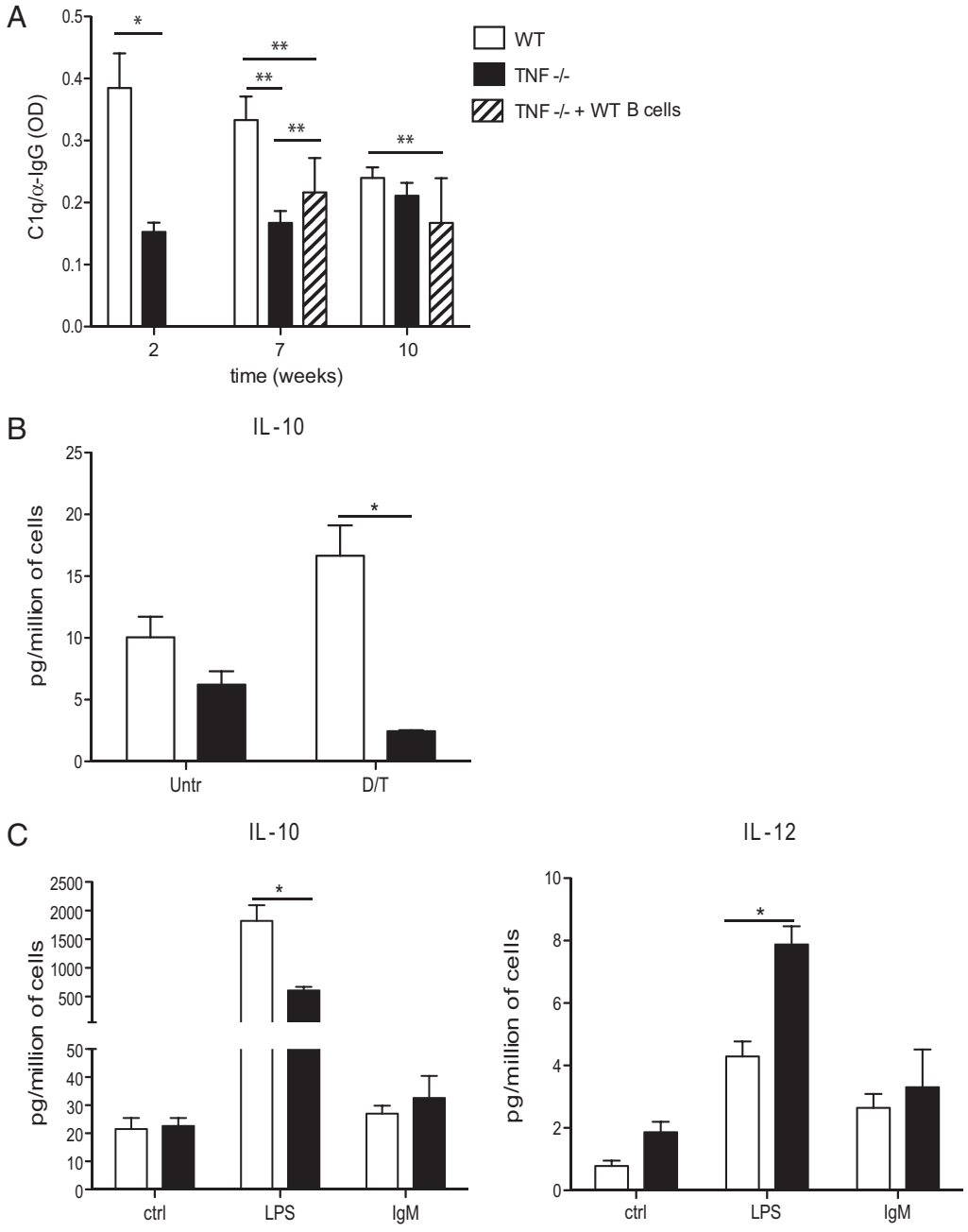

Fig. 3. Study of the activation of wild-type and $Tnf^{-/-}$ B cells. (*A*) C1q/IgG immune complexes in serum of wild-type mice ($n = 4$), $Tnf^{-/-}$ mice ($n = 4$), and in mice with $Tnf^{-/-}$ and wild-type B cells ($n = 3$). Immune complexes are reduced in $Tnf^{-/-}$ mice and are slightly increased after transfer of wild-type B cells but only at the 7-wk time point. $*P < 0.05$; $**P < 0.001$). (*B*) IL-10 production by lymphocytes from wild-type ($n = 6$) and $Tnf^{-/-}$ ($n = 6$) inguinal lymph nodes. Mice were treated with DMBA and then TPA for 4 wk. Lymphocytes were kept in culture for 24 h, and cytokine expression was measured by electrochemiluminescence. $*P < 0.05$. (*C*) IL-10 and IL-12 total production by wild-type ($n = 6$) and $Tnf^{-/-}$ ($n = 6$) B cells after 48 h incubation with LPS (10 μg/mL) and goat F(ab)2 anti-mouse IgM (10 μg/mL). $*P < 0.05$. Error bars indicate SE.

B cell-deficient mice (Fig. 1*B*) and the observation that B cells do not fully rescue papilloma development in $Tnf^{-/-}$ mice suggest that other leukocyte lineages also may contribute to papilloma development. After DMBA/TPA treatment, $CD4^{-/-}$ mice develop fewer papillomas than wild-type C3H/HeN mice (29), and we also have found $CD4^{+}$ cells to be involved in the tumor-promoting actions of TNF-α in ovarian cancer (11). In paper described above (29), it was reported that $CD8^{-/-}$ mice developed more papillomas, which would fit with the data presented here.

Our results may have broader significance for cancer-related inflammation. B regulatory cells suppress harmful autoimmune responses during chronic inflammation via IL-10 that efficiently dampens T-helper type 1–mediated processes (30). However, an unwanted outcome may be that the host immune system fails to recognize and destroy cells that have acquired oncogenic mutations. Against a background of chronic inflammation, initiated cells survive and proliferate, establishing premalignant and malignant lesions.

Materials and Methods

Mice. In each experiment, 6-wk-old wild-type, $Tnf^{-/-}$, *Jh*, $Rag2^{-/-}$, and CD19-Cre/$Tnf^{f/f}$ mice were age matched to within 3 d. $Tnf^{-/-}$, $Rag2^{-/-}$, and CD19-Cre/

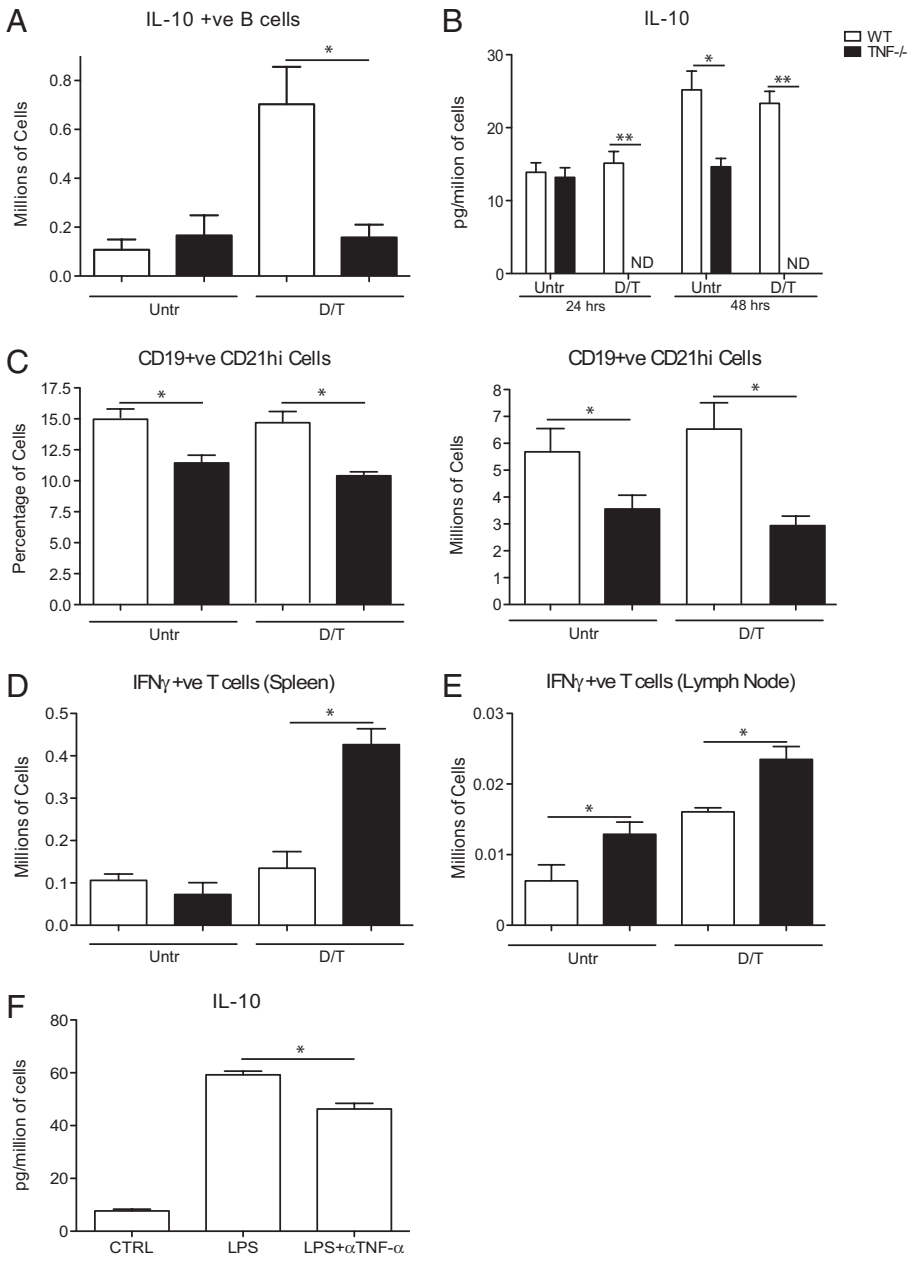

Fig. 4. Characterization of B-cell subpopulations in wild-type and *Tnf*$^{-/-}$ mice. (*A*) B cells (10^6) from untreated and DMBA/TPA-treated (5 wk) spleens of wild-type and *Tnf*$^{-/-}$ mice (n = 6) positive for IL-10. *P < 0.05. (*B*) IL-10 production by B cells from untreated and DMBA/TPA-treated (5 wk) spleens of wild-type and *Tnf*$^{-/-}$ mice (n = 6) after 24 and 48 h in culture. *P < 0.05; **P < 0.001. ND, not detectable. (*C*) Percentage (*Left*) and absolute numbers (*Right*) of CD19$^+$veCD21^{hi} cells from untreated and DMBA/TPA-treated (5 wk) spleens of wild-type and *Tnf*$^{-/-}$ mice (n = 6). *P < 0.05. (*D*) Absolute numbers of CD3$^+$ T cells expressing IFN-γ from untreated and DMBA/TPA-treated (5 wk) spleens of wild-type and *Tnf*$^{-/-}$ mice (n = 6). *P < 0.05. (*E*) Absolute numbers of CD3$^+$ T cells expressing IFN-γ from untreated and DMBA/TPA-treated (5 wk) inguinal lymph nodes of wild-type and *Tnf*$^{-/-}$ mice (n = 6). *P < 0.05. (*F*) IL-10 production by B cells from untreated spleens of wild-type mice (n = 6) after 6 h in culture with medium only (Control), LPS (10 μg/mL), or LPS (10 μg/mL) plus anti–TNF-α (10 μg/mL). *P < 0.05. Error bars indicate SE.

Tnf$^{f/f}$ mice were kept on a C57/Bl6 J background. *Jh* mice were on a BALB/c background and were provided by Taconic.

Skin Carcinogenesis. A dorsal area of skin 1–2 cm^2 was shaved, and 25 μg DMBA or 4μ TPA dissolved in 100 μL acetone was administered by micropipette. The area was shaved regularly, and the mice were observed daily. Tumors were assessed weekly and were defined as raised lesions with a minimum diameter of 1 mm that had been present for at least 2 wk.

Adoptive Transfer of B Lymphocytes. Spleens from wild-type or *Tnf*$^{-/-}$ mice (after 4 wk of TPA applications) were homogenized through a 70-μm nylon filter (Falcon). Single-cell suspensions were treated with 1× PharM Lyse ammonium chloride lysing reagent (BD Biosciences) for 5 min to remove erythrocytes. CD19$^+$ B cells were purified by positive selection using CD19 microbeads (catalog no. 130–052-201; Miltenyi Biotec) according to the manufacturer's instructions. Purity typically was >98% positive as controlled by FACS. B cells (7×10^6) were adoptively transferred into *Rag2*$^{-/-}$ mice or *Tnf*$^{-/-}$ mice in 200 μL of PBS by tail vein injection. When B cells were transferred into *Rag2*$^{-/-}$ mice, the mice were injected twice (on the day of transfer and 1 d after transfer) with 200 μg of purified GK1.5 antibody to avoid potentially contaminating CD4$^+$ T cells after adoptive transfer.

Immunohistochemistry. Age-matched tissue samples from *Tnf*$^{-/-}$ mice, wild-type mice, and mice with *Tnf*$^{-/-}$ plus wild-type B cells were immersion-fixed in 4% neutral-buffered formalin followed by dehydration through a graded series of alcohols and were embedded in paraffin. Tissue sections (5-μm thick) were deparaffinized using xylene and were rehydrated through a graded series of alcohol and subjected to enzyme and immunohistochemical detection as previously described (31). To detect macrophages, a specific marker, F4/80, was visualized on paraffin-embedded tissue sections. For antigen retrieval, Citra buffer (Vector Laboratories) was used. Nonspecific binding was blocked using PBS containing 5% goat serum (Thermo Fisher) and 2.5% BSA (blocking buffer). Sections were incubated overnight with rat anti-mouse F4/80 (1:100; Insight Biotechnologies) in 0.5× blocking buffer at 4 °C. Sections then were incubated with biotinylated rabbit anti-rat (1:200; Vector Laboratories) for 45 min at room temperature. Slides subsequently were incubated with HRP-conjugated avidin complex (ABC Elite; Vector Laboratories) for 30 min, followed by incubation with Fast 3,3 diaminobenzidine (DAB) (Vector Laboratories). Sections were counter-

stained with hematoxylin and mounted with depex mounting medium (Fisher Scientific).

To detect $CD8^+$ T cells, we used frozen sections. Samples were fixed in cold chloroform for 5 min and then dried for 5 min. Nonspecific binding was blocked using PBS containing 5% goat serum (Thermo Fisher) and 2.5% BSA (blocking buffer). Sections were incubated with rat anti-mouse CD8 (1:100; BD Pharmingen) in 0.5× blocking buffer overnight at 4 °C. Sections then were incubated with biotinylated rabbit anti-rat (1:200; Vector Laboratories) for 45 min at room temperature. Slides subsequently were incubated with HRP-conjugated avidin complex (ABC Elite; Vector Laboratories) for 30 min, followed by incubation with DAB (Vector Laboratories). Sections were counterstained with hematoxylin and mounted with depex mounting medium.

All immunolocalization experiments were repeated on multiple tissue sections and included negative controls for determination of background staining, which was negligible. Photographs were captured at high magnification (40×) using Image-Pro Plus 5.1 with a Penguin 150Cl camera (Pixera) coupled to an Eclipse 80i microscope (Nikon). Quantitative analysis of innate immune cells was performed by counting cells in 10 high-power fields (40×) per age-matched tissue section from three or five mice per group. Data presented reflect the mean total cell count per field.

Isolation of mRNA and Analysis by Real-Time RT-PCR. RNA was extracted from interfollicular epidermis and abraded skin using solution D as described previously (32). Interfollicular epidermis from three or four mice was pooled for each sample. Multiplex real-time RT-PCR analysis was performed using the target primers and probe (FAM) (CCL5: Mm01302427; IFN-γ: Mm00801778; designed with Primer Express) and 18s rRNA primer and probe (VIC) with the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems).

Immunoassay for CIC. To analyze CIC, ELISA was performed on purified serum using the CIC Mouse ELISA Kit (Alpha Diagnostic) according to manufacturer's instructions. The method is based on the specific binding of C1q to immune complexes, followed by a secondary step in which application of anti-IgG antibodies confirms the presence of CIC. To verify the presence of CIC in serum, a high-salt confirmation solution was added to dissociate C1q–CIC binding. Serum samples displaying 30% decrease in absorbance after the addition of the confirmation solution were considered positive for the presence of CIC.

Electrochemiluminescence Detection. Cytokines from supernatant of cells from inguinal lymph nodes (Fig. 3) or B cells (Figs. 3 and 4) were estimated using electrochemiluminescence detection with the Meso Scale Discovery assay according to the manufacturer's protocol. After defrosting at 4 °C, the calibrator standard and supernatant samples were incubated on the Meso Scale Discovery microplates using the Mouse TH1/TH2 9-PlexUltra-Sensitive Kit (K15013C-2). Plates were washed and read using SECTOR Imager 2400 software (Meso Scale Discovery).

In Vitro B-Cell Stimulation. B cells were purified as described above and then were kept in culture at 10^6 cells/mL in complete medium with 10 μg/mL LPS or 10 μg/mL goat F(ab)2 anti-mouse IgM (mu) (M31600; Caltag) for 24 or 48 h or were stimulated with 10 μg/mL LPS or 10 μg/mL LPS and 10 μg/mL anti–TNF-α for 6 h. Then the supernatant was collected for analysis of cytokines.

Flow Cytometric Analysis of Intracellular Cytokine Synthesis. Intracellular cytokine analysis was performed as previously described (33). Briefly, splenocyte or lymph node cultures were suspended at 5×10^5 cells/mL in complete medium with PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (500 ng/mL) (Sigma-Aldrich), and GolgiPlug (BD Biosciences) for 5 h. Cells then were stained extracellularly, followed by permeabilization and incubation with anti-mouse IL-10 or IFN-γ allophycocyanin-conjugated mAbs. The cells were acquired with a FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Statistical Analysis. Statistical analysis of experiments used the Wilcoxon–Mann–Whitney and log rank test or unpaired Student *t* test (PRISM 5; GraphPad Software Inc.). *P* values <0.05 were considered statistically significant.

1. Moore R, et al. (1999) Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 5:828–831.
2. Suganuma M, et al. (1999) Essential role of tumor necrosis factor α (TNF-α) in tumor promotion as revealed by TNF-α-deficient mice. *Cancer Res* 59:4516–4518.
3. Pikarsky E, et al. (2004) NF-κB functions as a tumor promoter in inflammation-associated cancer. *Nature* 431:461–466.
4. Maeda S, Kamata H, Luo JL, Leffert H, Karin M (2005) IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 121:977–990.
5. Kulbe H, et al. (2007) The inflammatory cytokine TNF-α generates an autocrine tumor-promoting network in epithelial ovarian cancer cells. *Cancer Res* 67:585–592.
6. Balkwill F (2009) Tumour necrosis factor and cancer. *Nat Rev Cancer* 9:361–371.
7. Popivanova BK, et al. (2008) Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* 118:560–570.
8. Arnott CH, et al. (2004) Expression of both TNF-α receptor subtypes is essential for optimal skin tumour development. *Oncogene* 23:1902–1910.
9. Hagemann T, et al. (2008) "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J Exp Med* 205:1261–1268.
10. Kim S, et al. (2009) Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature* 457:102–106.
11. Charles KA, et al. (2009) The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J Clin Invest* 119:3011–3023.
12. de Visser KE, Korets LV, Coussens LM (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 7:411–423.
13. Andreu P, et al. (2010) FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* 17:121–134.
14. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G (1996) Immune and inflammatory responses in TNF α-deficient mice: A critical requirement for TNF α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J Exp Med* 184:1397–1411.
15. Pasparakis M, et al. (1997) Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor. *Proc Natl Acad Sci USA* 94:6319–6323.
16. Kuprash DV, et al. (2005) Novel tumor necrosis factor-knockout mice that lack Peyer's patches. *Eur J Immunol* 35:1592–1600.
17. Grivennikov SI, et al. (2005) Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: Protective and deleterious effects. *Immunity* 22:93–104.
18. de Visser KE, Eichten A, Coussens LM (2006) Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* 6:24–37.
19. Tan TT, Coussens LM (2007) Humoral immunity, inflammation and cancer. *Curr Opin Immunol* 19:209–216.
20. Su TT, Guo B, Wei B, Braun J, Rawlings DJ (2004) Signaling in transitional type 2 B cells is critical for peripheral B-cell development. *Immunol Rev* 197:161–178.
21. Mauri C, Ehrenstein MR (2008) The 'short' history of regulatory B cells. *Trends Immunol* 29:34–40.
22. Evans JG, et al. (2007) Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol* 178:7868–7878.
23. Bouaziz JD, Yanaba K, Tedder TF (2008) Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol Rev* 224:201–214.
24. DiLillo DJ, Matsushita T, Tedder TF (2010) B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Ann N Y Acad Sci* 1183:38–57.
25. Biswas SK, Mantovani A (2010) Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nat Immunol* 11:889–896.
26. Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M (2010) B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature* 464:302–305.
27. Brodt P, Gordon J (1978) Anti-tumor immunity in B lymphocyte-deprived mice. I. Immunity to a chemically induced tumor. *J Immunol* 121:359–362.
28. Inoue S, Leitner WW, Golding B, Scott D (2006) Inhibitory effects of B cells on antitumor immunity. *Cancer Res* 66:7741–7747.
29. Yusuf N, et al. (2008) Antagonistic roles of CD4+ and CD8+ T-cells in 7,12-dimethylbenz(a)anthracene cutaneous carcinogenesis. *Cancer Res* 68:3924–3930.
30. Mauri C, Blair PA (2010) Regulatory B cells in autoimmunity: Developments and controversies. *Nat Rev Rheumatol* 6:636–643.
31. Coussens LM, Hanahan D, Arbeit JM (1996) Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *Am J Pathol* 149:1899–1917.
32. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
33. Mauri C, Gray D, Mushtaq N, Londei M (2003) Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197:489–501.