Lymphocyte-specific TRAF3-transgenic mice have enhanced humoral responses and develop plasmacytosis, autoimmunity, inflammation, and cancer.

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SUMMARY

TNF-Receptor Associated Factor 3 (TRAF3) regulates both innate and adaptive immunity by modulating signaling by Toll-Receptors (TLR) and TNF-Receptors. TRAF3 was recently identified as a tumor suppressor in human multiple myeloma, suggesting a prominent role in plasma cell homeostasis. We have generated transgenic mice expressing human TRAF3 in lymphocytes. These mice are normal at birth, but they develop over time plasmacytosis and hypergammaglobulinemia, as well as systemic inflammation and tertiary lymphoid organ formation. The analysis of the humoral responses of the TRAF3 mice demonstrated increased responses to T-dependent and T-independent antigens with increased production of antigen-specific IgGs compared to wild-type mice. Furthermore, TLR-mediated IgG production is also increased in TRAF3 B cells. In addition, TRAF3 mice develop autoimmunity and are predisposed to cancer, particularly squamous cell carcinomas of the tongue (≈50% incidence) and salivary gland tumors. In summary, TRAF3 renders B-cells hyper-reactive to antigens and TLR agonists, promoting autoimmunity, inflammation, and cancer, hereby providing a new model for studying de novo carcinogenesis promoted by B-cell-initiated chronic inflammation.
INTRODUCTION

TNF-Receptor Associated Factors (TRAFs) constitute a family of adapter proteins that interact with the cytosolic regions of multiple Tumor Necrosis Factor (TNF)-family receptors (TNFRs) upon their activation. TRAFs function as docking molecules for proteins involved in TNFR signaling. Furthermore, most TRAFs also catalyze ubiquitination of various target proteins via their intrinsic E3 ubiquitin ligase activity, stimulating substrate conjugation with either lysine 48- or lysine 63-linked poly-ubiquitin chains, with differing consequences in terms of proteasome-dependent protein degradation and protein activation, respectively \(^1-3\).

TRAF3 is among six members of this family of proteins in humans and mice, and has been shown to interact with several members of the TNFR family (reviewed in \(^3,4\)). Unlike many other TRAF-family proteins that enhance NF-κB activation, TRAF3 has been reported to suppress TNFR family-induced NF-κB activation \(^5\) and was identified as a negative regulator of NF-κB inducing kinase (Nik), promoting its degradation \(^6\). Consistent with an antagonistic effect of TRAF3 on the alternative NF-κB activation pathway, p100 NF-κB2 deficiency rescues mice from lethality caused by TRAF3 gene ablation \(^7\). Moreover, recent results indicate that mice with TRAF3 deficiency targeted to B-cells develop splenomegaly and lymphadenopathy, with hyperglobulinemia and autoimmunity \(^8\), suggesting a role for TRAF3 in B-cell homeostasis. In this regard, a tumor suppressor role for TRAF3 has been revealed in human multiple myeloma (MM). Indeed, mutations resulting in homozygous \(TRAF3\) gene inactivation have been found in 4-12% of these plasma cell malignancies \(^9,10\).

TRAF3 has also been identified as a key regulator of innate immunity, by participating in Toll-Like Receptor (TLR)-mediated responses to pathogens \(^11-14\). Furthermore, TLR-function is also required for B-cell responses to T-cell-dependent (TD) antigens \(^15\), as well as for
germinal center formation and plasma cell differentiation, which suggests that TRAF3 may participate in the regulation of TLR-mediated B-cell-responses.

In this report, we describe the generation of lymphocyte-specific TRAF3 transgenic mice. These mice over-express TRAF3 in B-cells, and develop hypergammaglobulinemia, plasmacytosis, autoimmunity, systemic inflammation, and cancer. These findings show a key role for TRAF3 in B cell homeostasis suggest that TRAF3 might indirectly promote carcinogenesis through B-cell initiated pro-inflammatory actions. The reported mouse model also provides the first example of solid tumors arising de novo in the setting B-cell initiated chronic inflammation without requirement for an exogenous carcinogen, thus mimicking human conditions associated with cancer risk in the setting of chronic inflammation and providing a novel animal model for testing chemopreventive strategies for head and neck cancers.

**EXPERIMENTAL PROCEDURES**

**Transgenic mice.** Lymphocyte-specific TRAF3-transgenic FVB/N mice were generated by randomly inserting a cassette encompassing full-length human TRAF3 cDNA under the control of the Vh8C4 promoter and the IgH µ enhancer (kindly provided by Dr. Hitoshi Kikutani, Osaka University). Analysis of the transgenic mouse genotypes was performed by PCR using primers specific for human TRAF3, and verification of the transgene expression was accomplished by immunoblotting using an anti-human TRAF3 polyclonal antibody. All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee. Euthanasia was performed according to the rules of the American Veterinarian Medical Association. Unless otherwise specified, all data shown
were generated using the ε-line of TRAF3 transgenic mice and their normal littermate controls.

**Cell isolation.** Cells were isolated from spleens, lymph nodes, and bone marrow (obtained from femurs). Mononuclear cells were isolated by Ficoll density-gradient centrifugation. B-cells were purified using the murine B-cell enrichment cocktail from Stem Cells technologies (Vancouver, Canada) following the manufacturer’s specifications. T-cells were purified using Mouse T-cell enrichment columns (R&D systems, Minneapolis, MN). Blood was collected from the cavernous sinus into tubes coated with heparin.

**Flow cytometry.** Lymphocyte suspensions were incubated with 50 µg/ml human γ-globulin to block Fc-receptors. Then 1-5 X 10^5 cells were incubated with a combination of allophycocyanin (APC)-, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies recognizing various surface markers (all from BD biosciences, San Jose, CA). After 1h incubation at 4°C, cells were washed and subjected to flow cytometry analysis (FACScanto, BD biosciences).

**Immunohistochemistry.** Tissues and organs from mice were fixed either in Bouin’s (Sigma, San Louis, MS) or zinc-buffered (Z-fix) (Anatech Ltd, Hayward, CA) solutions, and embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) or used for immunohistochemistry as described 16.

**Immunoblotting.** Cell lysates from frozen mouse tissues were prepared in modified Laemmli buffer and subjected to SDS-PAGE followed by immunoblot analysis as described 17. Anti-human TRAF3 16 was previously described. Antibodies against XBP-1, TRAF3 (C-20), and TRAF2 were from Santa Cruz Technologies (Santa Cruz, CA). Anti-IκBα was from Cell Signaling Technology (Danvers, MA). Anti-mouse Bcl-2 was from BD Biosciences. The
light chain of immunoglobulin was detected using HRP-labelled anti-mouse Ig antibodies (Biorad).

**Cytokine and autoantibody detection.** Mouse cytokines were quantified using the mouse Inflammatory Bead Array (CBA) kit (BD Biosciences). C-reactive protein (CRP) was detected using a mouse CRP ELISA kit (Kamiya Biomedical Company, Thousand Oaks, CA). Autoantibodies against dsDNA (Kamiya Biomed), nuclear antigens (ANA), ssDNA, and SSA/Ro (Alpha diagnostic International, San Antonio, TX) were quantified by ELISA.

**Serum immunoglobulins.** The amounts of IgM, IgG1, IgG2a, IgG2b in serum from TRAF3 and wild-type mice were determined by isotype-specific ELISA. Briefly, antibodies against mouse IgM, IgG1, IgG2a, and IgG2b (0.25 µg per well/50µl) were added to a 96 well plate (Nunc-Immuno plate, MaxiSorp surface, Nalge Nunc International, Denmark) and incubated overnight. Wells were washed with PBS and blocked with PBS containing 0.05% Tween20. Sera dilutions were added to the wells and incubated for 3 h. After extensive washing with PBS, AP-labeled anti-mouse IgM, IgG1, IgG2a, and IgG2b antibodies recognizing different complementary epitopes were added to the wells and incubated for 2h. Wells were washed 3 times with PBS, then isotype-specific immunoglobulins were quantified by colorimetric assay (AP-substrate kit, Biorad, Hercules, CA). All antibodies used for these experiments were from Southern Biotech (Birmingham, AL)

**Antigen challenges.** TRAF3 mice and wild-type littermates (3-6 months old) were inoculated either with 25 µg TNP-BSA in complete Freund’s adjuvant (followed 7 days later with the same inoculum, except with incomplete adjuvant) or with 25 µg TNP-LPS. Production of anti-TNP antibodies was determined by ELISA using TNP-KLH (0.25µg/well) as trapping antigen and detection of isotype-specific anti-TNP antibodies was achieved using alkaline phosphatase (AP)-labeled anti-IgM, anti-IgG1, and anti-IgG2a polyclonal
antibodies (Southern Biotech, Birmingham, AL). TNP-antigens were kindly provided by Dr. Ian Zitron (William Beaumont Hospital, Royal Oak, MI)

**TLR activation and immunoglobulin production.** Purified B-cells were cultured in RPMI containing 10% FCS, 1 mM L-Glutamine, antibiotics, oxalacetate, pyruvate and insulin (OPI) supplement (Sigma), and 50 μM 2-mercaptoethanol. B-cells were seeded in 12 well plates (2x10^5 cells/well), then either left untreated or activated with LPS (2 µg/ml) or with CpG (ODN1826, 1µM) (both from Invivogen, San Diego, CA). Culture supernatants and cells were collected at various times. The quantification of secreted IgM, IgG1 and IgG2a was performed, as described above.
RESULTS

Generation of mice over-expressing TRAF3 in lymphocytes.

We produced transgenic mice expressing full-length human TRAF3 under the control of a Vh promoter and an IgH μ-chain enhancer (Figure 1A). Three TRAF3 founders (termed α, ε and μ) showing stable expression of the TRAF3 transgene were obtained (supplementary Fig. 1A), with the ε-line showing the highest levels of expression as determined by immunoblot analysis of human TRAF3 protein levels. We therefore focused our analysis on this transgenic line. Expression of the TRAF3 transgene was restricted to the lymphoid lineage (Figure 1B). TRAF3 mice express the TRAF3-transgene predominantly in B-lymphocytes, although expression was also detected in a small subset of thymocytes and T lymphocytes (Fig 1C and not shown). Analysis of total levels of TRAF3 (endogenous + transgenic) in purified B-cells from spleens of TRAF3 heterozygous mice, by immunoblotting, showed that levels of TRAF3 protein in transgenic B-cells are 3-7-fold (average 4.8) elevated compared to B-cells from non-transgenic (wild-type [WT]) mice (Fig 1D). However, this increase in TRAF3 expression is within physiologic levels, since similar amounts of TRAF3 protein are produced upon B-cell activation ((Fig 1E) and 16). Furthermore, the levels of TRAF3 in our mice are similar to those observed in B-cells from a transgenic mouse model lacking functional TRAF2 (TRAF2DN) (Fig 1D) 18. In this regard, increased levels of TRAF3 have also been reported in TRAF2-deficient B-cells 19, in agreement with a role for TRAF2 in regulating TRAF3 degradation 20.

Mice over-expressing TRAF3 have reduced life-span and develop cachexia.

TRAF3 mice are born with normal Mendelian frequencies, lacking any gross abnormalities at birth. However, the survival of TRAF3 mice is reduced compared to wild-type littermates
Interestingly, TRAF3 mice develop an age-dependent phenotype characterized by oily hair (which becomes evident 2-3 months after birth) and cachexia, (weight loss starting 8-12 months after birth), becoming progressively runted as they age. A comparison of wild-type and runted TRAF3 littermates is shown in Supplementary Figures 2A and B. Weight loss affects both males and females (supplementary Figure 2C), though affecting females more severely (supplementary Figure 2D). TRAF3 mice with this phenotype lack body fat (Supplementary Fig 2B), have reduced muscular mass, and show severe reduction of organ sizes and weights, including liver, kidneys, lungs, heart (not shown) and spleen (Figure 1G and supplementary Figs 2E and F). The reduction in spleen size is age-dependent, since younger TRAF3 mice did not show significant differences in spleen weight compared to wild-type littermates (Fig 1G and Suppl. Figure 2E). Similar phenotypes were observed in mice bred into Fvb/n and mixed Fvb/n x BALB/c backgrounds (not shown).

TRAF3 mice have decreased B-cells but develop plasmacytosis and hypergammaglobulinemia.

Analyses of lymphocyte populations in spleens, lymph nodes (LN), and bone marrows (BM) from older TRAF3 mice showed a substantial increase in the ratio of T-cells versus B-cells (Suppl. Figure 3A). Total numbers of B-lymphocytes (B220⁺) were reduced in older TRAF3 mice, while the number of T-cells remained similar to wild-type littermates (Suppl. Fig 3B). Nonetheless, the ratio of mature (IgDH⁺) and immature (IgMH⁺) B-lymphocytes was not altered in TRAF3 mice compared to wild-type littermates (Suppl. Fig. 3C).

Interestingly, even in the earlier stages of the disease, TRAF3 showed significant increases in plasmablast/plasma cell populations compared to wild-type littermates (Figure 2A).
expanded plasma cell population in spleen and lymph nodes from TRAF3 mice expressed B220\textsuperscript{low to null}/IgM\textsuperscript{low to null} and high levels of syndecan-1 (Figure 2A) and cytosolic IgG (not shown).

In agreement with this result, immunohistochemical analyses of spleen and lymph nodes of TRAF3 mice revealed severe plasmacytosis. Mature plasma cells, as well as less mature plasmacytoid or plasmablastic cells, were abundant in medullary cords and paracortex of nodes (Figure 2B). Furthermore, histological analysis of various tissues and organs revealed generalized plasma cell infiltration (Figure 2C). The accumulation of plasma cells was not limited to the ε-line of TRAF3 transgenic mice. Though far fewer animals were analyzed, two other lines of TRAF3 transgenic mice derived from independent founders (α- and µ-lines) also showed evidence of plasmacytosis or plasmadyscrasias at frequencies higher than control mice. Comparison of the α-, ε- and µ-lines suggests a rough correlation between levels of TRAF3 transgene expression and the frequency of animals showing abnormalities in plasma cell homeostasis (Supplementary Figure 1A).

Immunoblot analysis of B-cells purified from TRAF3 transgenic mice (ε-line) showed high levels of XBP-1 protein, typical of mature plasma cells \textsuperscript{21}, as well as increased levels of IgG (Figure 2D) in TRAF3 mice. The increase in tissue plasma cells was accompanied by hypergammaglobulinemia in TRAF3 mice, which contained increased serum levels of IgG\textsubscript{2a} and IgG\textsubscript{2b}, while serum IgM levels remained similar in both control and TRAF3 littermates (Figure 2E). Hypergammaglobulinemia was age-dependent, and not observed in younger mice (not shown). Moreover, high levels of IgG\textsubscript{2a} and IgG\textsubscript{2b} were simultaneously present in TRAF3 mice, suggesting that plasmacytosis is the result of polyclonal expansions of plasma cells, and not a neoplastic process. Thus, TRAF3 appears to promote plasma cell accumulation in vivo.
Immunohistochemical analysis of several tissues from the TRAF3 mice also showed infiltrating immune cells organized into follicles, sometimes with germinal centers. These ectopic lymphoid structures were frequently found in pancreas (Figure 3A), kidney, liver, lung, and in mesentery and gut serosa (not shown) of TRAF3 mice. In contrast, ectopic lymphoid structures were never found in wild-type littermates. Both B and T lymphocytes were abundantly represented within these ectopic lymphoid accumulations (Figure 3B and C), indicating a reactive rather than neoplastic process. Surrounding cuffs of hematopoietic cells composed of lymphocytes (Figure 3D), as well as macrophages (histiocytes), mast cells, and plasma cells were also present within or in adjacency to the lymphoid aggregates, in addition to inflammatory cells dispersed through several organs in TRAF3 mice (data not shown).

**Elevated levels of inflammatory cytokines in the serum of TRAF3 transgenic mice.**

Levels of inflammatory cytokines were significantly elevated (p < 0.05) in serum of TRAF3 transgenic mice compared to age-matched wild-type animals, including TNF, MCP-1 and IFN-γ, consistent with a systemic inflammatory disorder rather than neoplasia. C-reactive protein was also significantly higher in TRAF3 transgenic mice (Figure 3F). Serum levels of IL-6, a known growth factor for plasma cells, were also higher in TRAF3 transgenic mice (37.4 + 3.6 pg/mL) compared to normal (29.4 + 9.1 pg/mL), but did not reach statistical significance (p = 0.20).

**TRAF3 mice develop autoimmunity.**

We measured autoantibodies in serum of mice. TRAF3 mice contained significantly higher concentrations of anti-nuclear antigens (ANA), (p=0.0012), anti-dsDNA Abs (p<0.0001), anti-ssDNA Abs (p=0.0013) and anti-SSA/Ro Abs (p=0.0012), compared to wild-type littermates (Figure 4A).
Histological analysis of kidneys from the TRAF3 mice also showed clear evidence of autoimmune kidney disease, involving primarily glomerular lesions but also tubulointerstitial changes (Figure 4B). Although the majority of the affected glomeruli were characterized by focal segmental damage, global lesions encompassing the total glomerular tuft were occasionally noticed (Fig 4B; panel a). Glomerular alterations included mesangial hypercellularity, mesangial matrix expansion with immunoglobulin deposition, glomerulonephritis with necrotizing and sclerosing lesions (Figure 4B; panel a-c), segmental necrosis associated with epithelial crescents, and alterations of peripheral capillary loops. Lobular accentuation and mesangialization of the peripheral capillary loops, associated with thickening of the peripheral capillary walls, were demonstrated by Jones’ silver methenamine (Fig 4B, panel d-e) and Masson’s trichrome (Fig 4B, panel f-g) stains. IgG (Figure 4B, panel h-i) and complement (C3) (Figure 4B, panel j-k) immunostainings visualized deposits confined to the mesangium or paramesangium or scattered in the subepithelial region. In addition, interstitial inflammation, fibrosis, and tubular epithelial changes were frequently present (Figure 4B; panel c). Pronounced renal pathology was found in 54% (14/26) of TRAF3 males [13-31 months old] and 51% (20/39) females [7-31 months old] but no wild-type animals. In contrast, occasional (5/23 [22%]) wild-type littermates had only very mild renal lesions, limited to scattered mononuclear cell infiltrations.

TRAF3 mice also develop arthritis (Figure 3E). In this regard, these mice had distended joint spaces, pannus formation, proliferation of synoviocytes, and irregular proliferation of cartilage and/or cartilage erosion at the junction of cartilage bone and synovium. In addition to joint involvement, foci of lymphocytic periductal infiltration were detected in the submaxillary and parotid glands (Fig 6F and G) of 5 of 27 (19%) male and in 1 of 30 (3%)
female TRAF3 mice. These inflammatory salivary gland lesions occurred in the absence of other oral cavity disease (see below), implying they arose *de novo* and were not secondary to trauma or malignancy. Altogether, these findings are consistent with development of severe autoimmune disease in TRAF3 mice, probably most consistent with lupus erythematosi, with Sjögren’s features.

Mice treated with pristane (2,6,10,14-tetramethyl-pentadecane) develop lymphoid neogenesis and autoimmunity \(^{22}\), as well as plasmacytoma on susceptible strain backgrounds \(^{23}\). Treating TRAF3 mice with pristane resulted in exacerbated inflammation, necrosis and ectopic lymphoid organ formation compared to untreated aged-matched TRAF3 mice and to pristane-treated wild-type littermates (not shown). However, pristane-treated TRAF3 mice bred onto the pristane-sensitive BALB/c background did not develop myeloma, indicating that TRAF3 over-expression in B-cells predisposes to inflammation but not myeloma or other B-cell malignancies.

**Enhanced humoral responses in TRAF3 mice**

Because TRAF3 mice have hypergammaglobulinemia and produce autoantibodies, we functionally assessed humoral immune responses in TRAF3 mice. For these experiments, TRAF3 mice and wild-type littermates were challenged with either TNP-BSA (T-dependent antigen) or TNP-LPS (T-independent antigen). IgM-specific responses to these antigens were similar in TRAF3 and wild-type mice (Figure 5A). However, IgG\(_1\) and particularly IgG\(_2\) responses to these antigens were significantly enhanced in TRAF3 mice. This enhanced antibody response was particularly evident in TRAF3 mice challenged with LPS, resulting in significantly higher amounts of antigen-specific IgG\(_{2a}\) and IgG\(_{2b}\) antibodies compared to wild-type littermates (Fig 5A). Thus, TRAF3 improves humoral responses to both TD and TI antigens.
TRAF3 B-cells are hyper-responsive to TLR activation.

A role for TRAF3 in innate immune responses has been previously suggested \(^{11,12}\). Growing evidence indicates that inappropriate activation of TLRs may be involved in development of lupus erythematosus and other autoimmune diseases \(^{24}\). Given that TRAF3 mice exhibited enhanced humoral responses to TLR agonists and develop autoimmunity, we assessed immunoglobulin production by B-cells activated with the TLR agonists LPS and CpG. TRAF3 B-cells activated with either LPS or CpG showed a striking increase in IgG\(_1\) and IgG\(_{2a}\) production compared to wild-type B-cells (Figure 5B). In contrast, LPS- and CpG-induced IgM production was similar for TRAF3 and wild-type B-cells.

To test whether hyper-responses to TLR were the result of accelerated B-cell proliferation, purified B-cells from TRAF3 and wild-type mice were culture in the presence of a variety of TLR agonists. TRAF3 and wild-type B-cells proliferate at similar rates in response to TLR activation (supplementary Figure 4A). These results suggest that TRAF3 does not affect TLR-mediated B-cell proliferation but rather regulates TLR-mediated B-cell differentiation.

TRAF3 over-expression reduces Nik, but does not alter CD40- or BAFF-R-mediated B-cell proliferation and signaling.

TRAF3 has been shown to interact with and regulate CD40 and BAFF-R \(^4\). Therefore, to determine whether TRAF3 over-expression alters the normal responses to these receptors, we first assessed whether TRAF3 over-expression in B-cells altered their proliferation in response to CD40 and BAFF-R activation, but found that TRAF3 B-cells proliferate at similar rates compared to wild-type B-cells in response to these agents (supplementary Figs. 4B,C), either alone or in combination with B-cell Receptor (BCR) activation (supplementary Figure 4D).
As expected, TRAF3 over-expression reduced endogenous levels of Nik (supplementary Figure 5A and B), in agreement with the previously described role of TRAF3 in controlling Nik degradation. The average levels of Nik expression in TRAF3 mice were 37±7% of wild-type mice. While starting with lower basal levels of Nik, stimulation with either CD40L or BAFF reduced Nik protein levels in B-cells from TRAF3 mice, similar to the wild-type B-cells (supplementary Figure 5A and B). TRAF3 expression in B-cells had no discernible effect on MAP3K activation (JNK, p38 and ERK), IκB degradation, p100 processing, p52 levels, or TRAF2 down-regulation in response to activation either with CD40L (supplementary Figure 5A) or BAFF (not shown) in TRAF3 B-cells compared to wild-type B-cells within the time frame examined here.

**TRAF3 mice are predisposed to cancer.**

Most TRAF3 mice (47 of 70 [67%]) developed solid tumors with aging. In contrast, only a minority of wild-type mice (3 of 24 [13%]) developed malignancies over the same time-frame (Figure 6A). The most frequent tumor developed by the TRAF3 mice was squamous cell carcinoma of the tongue (Figure 6B-C), which was found in 50% (19 of 38) of TRAF3 mice (40% of TRAF3 males (n=15) and 57% of TRAF3 females (n=23)). Examples of these cancers are shown in Figures. 6D and E. Tongue tumors were invaded by inflammatory cells, predominantly granulocytes and macrophages, with occasional plasma cells. Mice with tongue tumors uniformly had large reactive submandibular lymph nodes. No tongue tumors were found in wild-type mice (n=11). Furthermore, tumors were also found in 43% of the parotid glands derived from TRAF3 females (6 of 14), but only in 1 of 11 (9%) of TRAF3 males (Figure 6H and I). Parotid tumors included pleiomorphic adenomas, cystadenomas, and spindle cell carcinomas. Lymphoid infiltration in the parotid glands was
also evident (Figure 6G). Thus, TRAF3 mice are predisposed to developing tumors of the oral cavity and salivary glands.

Lung cancers (Figure 6J) were found in 27% (17 of 63) of TRAF3 mice (39% of TRAF3 males [n=28] and 17% of TRAF3 females [n=35]), comprised of 15 papillary bronchiolo-alveolar carcinomas (Figure 6J) and 2 adenomas, while only 14% (n=22) of wild-type littermates developed lung cancers. Interestingly, inflammatory lesions such as perivascular cuffing were frequently found in the vicinity of these solid tumors in TRAF3 mice. Moreover, peribronchial mononuclear cell infiltrations, lymphocytic interstitial pneumonitis, and occasional pleuritis were also commonly observed in TRAF3 mice (not shown). Altogether, inflammatory pulmonary lesions were present in 38% (n=63) of TRAF3 mice (18% [n=28] of TRAF3 males and 54% [n=35] of TRAF3 females), while lung tumors were present in 27% (n=63) of these mice, suggesting that lung inflammation commonly accompanies the development of lung cancer in these mice.

Hepatocellular carcinoma (Figure 6K) was found in 18% (n=38) of female TRAF3 mice, but none of the TRAF3 males (n=27) or wild-type littermates (n=22). Inflammatory changes, such as portal/periportal or perisepetal inflammation with lymphocyte aggregates (chronic hepatitis), focal lytic necrosis, and peliosis hepatitis were found in 22% (n=27) of TRAF3 males and 44% (n=34) of TRAF3 females (Figure 6H). In contrast, the only liver alteration found in wild-type mice was microgranuloma (9%, n=22), which are common in mice and are not necessarily associated with liver pathology.
DISCUSSION

TRAF3 was recently described as a tumor suppressor candidate in human multiple myeloma (MM), implying an important role for this adapter protein in B-cell and plasma cell biology. As many as 4-12% of MM specimens and 17% of MM cell lines harbor homozygous deletions or inactivating mutations in their *traf3* genes \(^9,10\). Mice lacking TRAF3 (*traf3/-*) in B-cells through tissue-specific gene ablation develop splenomegaly and lymphadenopathy, as well as hyperglobulinemia and autoimmunity \(^8\), consistent with the idea that elimination of TRAF3 removes an obstacle to B-cell expansion in vivo. In contrast to mice with *traf3/-* B-cells (e-line), our animals over-expressing TRAF3 in B-cells developed hypotrophy of spleen with aging, and decreased numbers of B-cells in vivo. Remarkably, TRAF3 transgenic mice develop hypergammaglobulinemia and severe plasmacytosis in spleen and lymph nodes, with frequent infiltration of plasmablasts and plasma cells in tissues. Thus, despite TRAF3’s potential to inhibit B-cell accumulation in vivo, plasma cell expansion is enhanced in TRAF3 transgenic mice, which seems at odds with a tumor suppressor role for TRAF3 in myeloma. These results strongly suggest a role for TRAF3 in promoting plasma cell differentiation, where the over-expression of TRAF3 in B-cells drives these cells towards a plasma cell phenotype. Conversely, however, the absence of TRAF3 does not prevent B-cells from differentiating into antibody-producing plasma cells, as indicated by development of hyperglobulinemia in mice harboring TRAF3-deficient B-cells \(^8\). Thus, TRAF3 is not required for B-cells to differentiation into plasma cells, though it may enhance this differentiation. Importantly, we have previously reported that endogenous TRAF3 is prominently expressed in normal plasma cells in both humans and mice \(^16\), supporting a physiological role for TRAF3 in plasma cell differentiation.
The apparent paradox that both TRAF3 over-expression and TRAF3 knock-out cause hyperglobulinemia and autoimmunity in vivo could reflect different roles for TRAF3 that result in a similar phenotype, but for different reasons. For example, TRAF3 deficiency may cause plasma cell expansion (and thus hyperglobulinemia) because it prolongs plasma cell survival, while TRAF3 over-expression may cause plasma cell expansion because it drives B-cell differentiation. In this regard, *traf3*-/- B-cells reportedly have increased survival *ex vivo*, which might account for in vivo plasma cell accumulation in *traf3*-/- mice. Conversely, we can eliminate a role for TRAF3 in controlling B-cell proliferation, in that B-cells from our TRAF3 transgenic mice exhibit normal proliferative responses in culture compared to wild-type B-cells when stimulated with several different stimuli, including CD40L, BAFF and TLR agonists, which is in agreement to what has been described for *traf3*-/- B-cells. Thus, TRAF3 seems to have complex effects on B-cell survival and differentiation, which should be considered in the context of TRAF3’s putative role as a tumor suppressor.

**Role of TRAF3 in the control of humoral responses.** TRAF3 loss of function and gain of function through genetic manipulations in B-cells result in increased in vivo immunoglobulin production and development of autoimmunity, as described above, suggesting that TRAF3 has a critical role in the control of tolerance. These seemingly contradictory results might underline two different mechanisms controlled by TRAF3. While lack of TRAF3 increases survival of B-cells and consequently dysregulates B-cell homeostasis, which might result in the expansion of auto-reactive B-cells and autoimmunity, TRAF3 over-expression in B-cells enhances humoral responses against TD and TI antigens, essentially improving antibody generating activity, presumably by promoting B-cell differentiation. Consistent with the hypothesis that different mechanisms underlie these
seemingly similar phenotypes of TRAF3 gain and loss of function, the time-course with which \textit{traf3}-/- and TRAF3 transgenic mice manifest their antibody-mediated pathologies differs. Thus, mice lacking TRAF3 in B-cells develop hyperglobulinemia and autoimmunity at young age, consistent with a role of TRAF3 in the control of B-cell homeostasis with immediate effects on immunoglobulin production. In contrast, mice over-expressing TRAF3 in B-cells develop hypergammaglobulinemia and autoimmune pathology at older ages (> 1 year), with younger TRAF3 transgenic mice showing no evidence of disease, consistent with a cumulative effect of TRAF3 over-expression in enhancing humoral responses during a life-time of antigenic exposure. Consistent with this interpretation, the ability of TRAF3 B-cells to mount enhanced humoral responses to antigens \textit{in vivo}, and upon TLR activation \textit{in vitro}, was already evident in young (3-6 months old), asymptomatic mice.

Another striking difference in \textit{traf3}-/- and TRAF3 transgenic mice is found in the primary (IgM) versus secondary (IgG) antibody responses. In this regard, TRAF3 transgenic mice that develop hyperglobulinemia have similar IgM titers compared to wild-type littermates. Moreover, IgM production in response to both TI and TD antigens \textit{in vivo} and to TLR activation \textit{in vitro} is similar in both TRAF3 and wild-type mice. In contrast to IgM, it is IgG titers that rise in TRAF3 mice with aging, and production of IgG, in particular IgG\textsubscript{2a} and IgG\textsubscript{2b}, is greatly enhanced upon immunization, especially with TI antigens and upon activation with TLR agonists. These results suggest that TRAF3 over-expression in the B-cell lineage does not regulate the initiation of the humoral response, but rather controls later stages of B-cell differentiation or the duration of antibody responses. In contrast, mice lacking TRAF3 in B-cells show increased immunoglobulin production in response to TI antigens, including IgM and IgG isotypes, which is more consistent with heightened antibody responses as a result of increased survival of antigen-challenged B-cells, rather
than an alteration in B-cell differentiation\(^8\), as elaborated above. Altogether, these results support the idea that TRAF3 has different functions at different stages of B-cell development.

Recent data suggest that TRAF3 is a key regulator of innate immunity, by participating in the control of TLR-mediated signal transduction pathways activated by pathogens\(^{11,12}\). Our results show that TRAF3 is indeed involved in TLR-mediated humoral responses, and that its over-expression in B-cells greatly enhances humoral responses to both TI and TD antigens. Furthermore these results are also generally in agreement with the recently described role for TLRs in the control of B-cell-dependent responses to T-cell-dependent antigens\(^{15}\), which showed that TLR signaling is required for germinal center formation and plasma cell differentiation. Thus, we favor the idea that TLR-mediated signaling (rather than BCR-mediated signaling) is the principal context in which TRAF3 over-expression in B-cells leads to heightened antibody responses and plasma cell expansion.

**B-cells, chronic inflammation, and cancer.** TRAF3 mice develop systemic inflammation, manifested by high levels of inflammatory cytokines in serum, elevated C-reactive protein, white blood cell infiltration into tissues, and ectopic lymphoid organ formation. Furthermore, TRAF3 mice also develop cancer with high incidence, in particular squamous cell carcinomas of the oral cavity. Though we cannot exclude a gene expression modifying role of transgene insertion into the mouse genome, we hypothesize that the frequent development of cancers in TRAF3 transgenic mice results from chronic inflammation. Abundant data have established that cancer is promoted by chronic inflammation\(^{26,27}\). In particular, it is well documented that chronic inflammation, irrespective of the underlying etiology, can lead to the development of squamous cell carcinomas\(^{28,29}\).
Interestingly, De Visser and coworkers\textsuperscript{30} using a transgenic model of enforced expression of human papillomavirus type 16 (HPV16) early-region genes in keratinocytes, have recently shown that B lymphocytes are required for establishing chronic inflammatory states that promote de novo carcinogenesis. Indeed, malignant transformation of epithelium seems to require the participation of B-cells and immunoglobulin depositions in premalignant lesions. The inflammatory alterations and cancer developed by the B-cell-specific TRAF3 mice may have a similar etiology. However, we have failed to consistently observe immunoglobulin depositions in the squamous cell carcinomas developed by the TRAF3 mice (not shown). While further research will be required to assess the role of immunoglobulin deposition in inflammation and cancer development in the TRAF3 transgenic mice, it is worth noting that these mice require not exogenous carcinogens or oncoproteins to develop tumors, representing the first model of \textit{de novo} carcinogenesis involving B-cell-initiated inflammation and provide a novel model for testing chemopreventive therapies aimed at oral cancers, a setting where premalignant lesions progress to full-blown malignancy with the assistance of chronic inflammation.

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AUTHORSHIP

Dr. Zapata contributed to all scientific aspects of the manuscript and wrote the paper. Dr. Krajewska contributed the immunohistochemical analyses. Dr. Llobet designed and performed research, and analyzed data. Dr. Lefebvre and C.L. Kress performed research. Dr. J.C. Reed contributed by designing research, analyzing data, and writing the paper. The authors declare no conflicting financial interest.
REFERENCES


FIGURE LEGENDS

Figure 1. Generation of TRAF3 transgenic mice. A. Schematic representation of the TRAF3 transgenic constructs. The various components of transgenic cassette are indicated. B. Immunoblot analysis of the hTRAF3 transgene expression in various tissues from the TRAF3 transgenic mice (ε-line) and wild-type littermates. hTRAF3 expression was detected using an antibody that specifically recognizes human TRAF3, but not mouse TRAF3. C. Analysis of TRAF3 and IκBα expression in total splenocytes and in affinity-purified B- and T- lymphocytes isolated from TRAF3 transgenic mice and wild-type littermates. The TRAF3 antibody used (Santa Cruz, C-20) recognizes both human and mouse TRAF3. The purity of B- and T-cells was >95%. D. Purified B-cells from spleens of the various transgenic mice indicated and wild-type littermates were lysed in Laemmli buffer and sonicated. Lysates were normalized for protein content and 25 µg were immunoblotted. Total TRAF3 and IκBα levels are shown. Vertical white lines have been inserted to indicate a repositioned gel lane. E. B-cells from wild-type mice were left resting or stimulated with LPS (2 µg/ml) for 16 or 72 hours. TRAF3 and Bcl-2 expression were analyzed by immunoblotting. Vertical white lines have been inserted to indicate a repositioned gel lane. F. Kaplan-Meier analysis of the life-span of wild-type and TRAF3 littermates (17 mice per group). Statistical significance was calculated by log-rank analysis. G. The weight of spleens from mice up to 8 months (TRAF3, n=7; wild-type, n=5) or over 16 months old (TRAF3, n=27; wild-type, n=19) was determined. Statistical significance was determined by unpaired t-test.

Figure 2. TRAF3 mice develop plasmacytosis. A. Phenotypic analysis of plasma cells. Submaxillary lymph nodes (LN) and spleen lymphoid populations were analyzed by three-
color flow cytometry in a representative 14 month old TRAF3 transgenic mouse (TRAF3) and wild-type littermate (WT). Total nucleated cells were stained for B220, Syndecan-1 and IgM surface expression and analyzed with no gate (top) or selecting the IgM null cells (bottom). Numbers indicate the percentage of cells in that gate. **B.** Analysis of plasma cells in lymph nodes. Immunohistochemical analysis of a representative lymph node from a diseased TRAF3 mouse showing severe plasmacytosis. Plasma cells were identified by cytosolic Ig staining. Bars, 100μm. **C.** Plasma cell infiltrates in tissues from TRAF3 mice. Immunohistochemical analysis of the tongue (left) and pancreas (right) of a representative diseased TRAF3 mouse (top and middle panels) and a wild-type littermate (bottom panels). Left, H&E (top and bottom) or anti-IgG (middle) staining of sections of the tongue. Right, H&E staining of infiltrating plasma cell in the pancreas. Bars, 50μm. **D.** Analysis of the expression XBP-1 and IgG in purified B-cells. B-cell extracts (20 μg) were analyzed by SDS-PAGE and immunoblotting. **E.** TRAF3 mice develop hypergammaglobulinemia. Serum IgG<sub>2a</sub>, IgG<sub>2b</sub> and IgM concentrations from TRAF3 mice (ε-line) and wild-type littersmtes (10-15 months old) were determined using isotype-specific ELISA. p values are indicated in the figure. Statistical significance was determined by unpaired t-test.

**Figure 3. TRAF3 transgenic mice develop systemic inflammation.** **A.** Histological analysis. H&E staining shows lymphoid neogenesis (tertiary lymphoid organ [TLO] formation) in omentum and pancreatic parenchyma partly replaced by inflamed adipose tissue in TRAF3 mouse. **B.** B-cells in TLO were stained with B220 mAb. **C.** T-cells in TLO were stained with anti-CD3 mAb. **D.** H&E staining of an inflammatory cuff surrounding renal artery with thickened tunica media. **E.** H&E staining of a joint from a representative TRAF3 mouse with arthritis. Note the distended joint spaces, pannus formation, proliferation of
synoviocytes, the irregular proliferation of cartilage at the junction of cartilage bone and synovium or cartilage erosion. Bars, (A-E) 100 µm. F. Analysis of pro-inflammatory proteins in serum. Sera from TRAF3 transgenic mice and wild-type littermates (10-15 months old) were analyzed to determine the concentrations of inflammatory cytokines and C-reactive protein. Statistical significance was determined by unpaired t-test (p values are indicated in figure). Hash marks indicate means.

**Figure 4. TRAF3 mice develop autoimmunity.** A. Analysis of circulating autoantibodies in TRAF3 mice and wild-type littermates. Blood from TRAF3 mice (12 to 17 months old) and from wild-type littermates was extracted for serum collection. The presence of autoantibodies in serum was determined by ELISA (anti-dsDNA, 1:150 dilution; anti-ANA, anti-ssDNA ad anti-SSA/Ro, 1:50 dilution). Statistical significance was determined by unpaired t-test. B. Pathological alterations in kidneys from TRAF3 mice. Morphological lesions encompass both glomerular (a-k) and tubulointerstitial (a,c) alterations, demonstrated by H&E (a-c), Jones’ silver methanamine (d-e), and Masson’s trichrome (f,g) stains. Depositions of IgG (h-i) and C3 complement (j,k) were detected by immunostaining. Bars, 50 µm.

**Figure 5. TRAF3 mice exhibit enhanced humoral responses to TD and TI antigens.** A. Mice were challenged with TNP-BSA (T-dependent antigen) in complete Freund’s adjuvant, or with TNP-LPS (T-independent antigen). Blood was collected before and after injection of TRAF3 mice (◊) and 4 wild-type littermates (♀) with antigens at the days indicated. Titers of anti-TNP IgM and anti-TNP IgG2 were determined by ELISA using TNP-KLH-coated plates. Data represent mean ± SEM; n=4. B. B-cell antibody responses to TLR agonists. B-cells were cultured in 12 wells plates (2x10^5 cells/ml) and either untreated or treated with 2 µg/ml
LPS or with 1 µM CpG (ODN1826). Culture supernatants were collected at various days and secreted IgM, IgG₁ and IgG₂a were quantified. Data represent mean ± SEM for TRAF3 mice (n=5, gray bars) and wild-type littermates (n=4, black bars).

**Figure 6. Cancer development in TRAF3 transgenic mice.**

**A.** Percentage of tumors found in wild-type mice (n=24; 17±1.5 months) and in TRAF3 mice (n=70; 18±0.8 months).

**B.** Graphic representation of percentage of mice bearing tumors in the tongue (squamous cell carcinoma n=19, papilloma n=2), other oral tumors (pleomorphic adenomas, cystadenomas and adenocarcinomas), lung carcinomas (papillary bronchiolo-alveolar carcinoma, n=15; adenoma, n=2), and hepatocarcinoma (n=7).

**C.** Comparative analysis of tongues from 2 month (bottom) and 14 month (top) old TRAF3 mice and wild-type littermates. The arrow indicates the squamous cell carcinoma in the tongue. **D and E.** Squamous cell carcinoma of the tongue (H&E).

**F.** and **G.** Example of lymphoid infiltration in submaxillary gland and parotid gland, respectively, in TRAF3 mice. **H.** and **I.** H&E staining of parotid glands with pleomorphic adenoma and sarcoma, respectively. **J.** Papillary bronchiolo-alveolar carcinoma of lung in a TRAF3 mouse. **K.** H&E staining of hepatocellular carcinoma in TRAF3 mouse. Note the presence of subcapsular fat necrosis with chronic inflammatory cells. Bars (D-K), 100 µm.
SUPPLEMENTARY FIGURES

Supplementary Fig 1.

A. Analysis of TRAF3 transgene expression in transgenic mice. Immunoblot analysis of the hTRAF3 transgene expression in spleen from three TRAF3 transgenic lines (TRAF3α, ε and µ) (+) and wild-type littermates (-). hTRAF3 expression was detected using an antibody that specifically recognizes human TRAF3, but not mouse TRAF3. B. Histological analysis of tissues from TRAF3 mice. Tissues from wild-type mice (n=20) and the three TRAF3 lines (TRAF3α (n=13), TRAF3ε (n=48) and TRAF3µ (n=3)) were fixed in Z-fix solution and embedded in paraffin. Tissue sections were stained with H&E and evaluated for evidence of plasmacytosis or plasmadyscrasias and inflammation. The percentage of animals displaying such pathology is indicated.
**Supplementary Fig 2.**

**Macroscopic assessment of phenotype of TRAF3 mice.** 

**A.** Photograph showing a comparison between a cachectic TRAF3 female mouse (top) and an age-matched wild-type female littermate (ε-line). 

**B.** Gross analysis of abdominal cavity of the mice shown in panel A. Note the absence of abdominal fat in the TRAF3 mouse. 

**C.** Analysis of the weights of wild-type and TRAF3 female (wt, n=9; TRAF3, n=11) and male (wt, n=10; TRAF3, n=11) mice between 12 and 15 month old. Statistical significance was determined by unpaired t-test. 

**D.** Graphic representation of the weight variation over a period of 10 months of wild-type and TRAF3 littermates. Only data from female mice were included in the figure, although TRAF3 male mice also suffer from weight loss. 

**E.** Comparative analysis of spleens from 2 months and 14 month old TRAF3 and wild-type mice. The 14 month old TRAF3 mouse was cachexic. 

**F.** Analysis of submaxillary gland lymph nodes and spleens from a cachexic 13 month old TRAF3 mouse and a wild-type littermate. Note the enlarged LN and reduced spleen of the TRAF3 mouse.
Supplementary Fig 2

A

B

C

D

E

F

WILD-TYPE

TRAFl3

WEIGHT (g)

P=5x10^-4

P=0.008

FEMALES

MALES

WEIGHT (%)

TRAF3 fem

wt fem

AGE (MONTHS)
Supplementary Figure 3.

Analysis of B-cell and T-cell populations from lymphoid organs isolated from TRAF3 transgenic mice and wild-type littermates. A. Single cell suspensions were prepared from spleens, lymph nodes (LN) and bone marrow (BM) from a 14 month old TRAF3 (ε-line) mouse suffering from weight loss (bottom) or from a wild-type littermate (top). Mononuclear cells were blocked with human γ globulin, and stained anti-B220 mAb and anti-CD3 mAb. FACS profiles of gated lymphocytes are shown in the figure. The percentage of lymphocytes in each quadrant is indicated. B. Lymphocytes from the spleens of the mice used in A were stained with anti-B220 mAb, anti-IgD and anti-IgM (left) or with anti-CD3 mAb, anti-CD4 and anti-CD8 (right). The IgM/IgD profile of the B220 positive population (left) and the CD4/CD8 profile of the CD3 positive population (right) are shown. C. Total number of splenocytes from TRAF3 transgenic (n=7) and wild-type mice (n=7) were enumerated. The percentage of B-cells (B220+) and T-cells (CD3+) in each sample were determined by FACS analysis and the total number of B-cells and T-cells was determined. Data represent mean ± SEM. Statistical significance was determined by unpaired t-test.
Supplementary Figure 4.

**TRAF3 over-expression does not alter B-cell proliferation.** For these experiments, mouse spleens were carefully crushed to release the lymphocytes. Cell suspensions were depleted of red cells and neutrophils by density centrifugation (Lympholite-M, Cedarlane laboratories, Hornby, Ontario). B-cells were purified using the murine B-cell enrichment cocktail from Stem Cells technologies (Vancouver, Canada). **A.** Effect of TLR-agonists on B-cell proliferation. Purified B-cells (5 x 10^4) from TRAF3 transgenic mice (n=4) and wild-type littermates (n=4) were incubated with Pam3CSK4 (1 µg/ml; TLR1/2 agonist), HKLM (108 cells/ml; TLR2 agonist), Poly(I:C) (25 µg/ml; TLR3 agonist), LPS (1 µg/ml; TLR4 agonist), Flagelin (2.5 µg/ml; TLR5 agonist), FLS1 (1 µg/ml; TLR2/6 agonist), ssRNA40 (2.5 µg/ml; mouse TLR7 agonist), ODN1826 (CpG, 2 µM; TLR9 agonist) (all from Invivogen, San Diego, CA) or 10 or 25 µg/ml of anti-µ mAb (F(ab)'2 fragment; Jackson Immunoresearch Lab. Inc. West Grove, PA) for 62h at 37 ºC. Then, ^3^H-Thymidine (0.5 µCi/well) was added and 10 hours later cells were harvested and ^3^H-thymidine incorporation into DNA was measured. No statistically significant differences were detected for TRAF3 versus WT mice (mean ± SEM; n=3). **B.** Effects of IL-4 and CD40L on B-cell proliferation. Purified B-cells (5 x 10^4) from TRAF3 transgenic mice and wild-type littermates (control) were incubated with IL-4 alone (4 ng/ml), CD40L alone (1 µg/ml) or a mixture of IL-4 (4 ng/ml) plus 100 ng/ml or 1 µg/ml of CD40L for 62 h. ^3^H-thymidine incorporation into DNA was subsequently measured as described above. Trimeric CD40L was kindly provided by Immunex Corporation (Thousand Oaks, CA). Data are mean ± SEM (n=3). **C.** Effect of BAFF on B-cell proliferation. Purified B-cells (n=3 for each genotype) were incubated with 100 ng/ml of purified BAFF (R&D systems, Minneapolis, MN) for 68 h. Then ^3^H-thymidine incorporation into DNA was measured as described above. Data represent
fold induction of DNA synthesis relative to unstimulated cells. **D.** Effect of BCR and BAFF on proliferation of B-cells. B-cells were cultured with or without anti-B-cell Receptor (BCR) reagent (1 µg/ml anti-µ mAb, F(ab)’2 fragment; Jackson Immunoresearch Lab. Inc. West Grove, PA) alone or in combination with 100 ng/ml of mouse BAFF (R&D systems). DNA incorporation was measured and data presented as fold-increase relative to unstimulated cells.
**Supplementary Figure 5.**

CD40-mediated signaling events in TRAF3 mice. A. TRAF3 transgenic B-cells have reduced Nik but most CD40-induced signal transduction events are intact. Purified B-cells (5x 10^6) from a pair of age-matched, sex matched wild-type and TRAF3 transgenic littermates were left untreated or treated with 1 µg/ml CD40L (R&D systems) for the indicated times (t). Cell lysates were prepared (10 µg total protein) and analyzed by SDS-PAGE/immunoblotting using various antibodies specific for TRAF3, TRAF2, IκBα and RelA (Santa Cruz Biotech. Santa Cruz, CA), JNK, phospho-JNK, ERK, phospho-ERK, p38, phospho-p38, p100/p52 NFκB2, Nik (Cell Signaling Technology, Danvers, MA), Tubulin (Sigma, San Louis, MO), and c-Rel (R&D Systems). Antibodies were detected by an ECL method. B. **Quantification of Nik protein levels in B-cells before and after CD40 activation.** B-cells were isolated from the spleens of control and TRAF3 transgenic mice (two mice each) and were left untreated or activated with CD40L as described above. Cell lysates were prepared and 10 µg total protein were analyzed by SDS-PAGE/immunoblotting. The Kodak Digital Science 1D (v 3.0) was used for densitometry.
Supplementary Figure 5

A

CD40L

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B

Supplemental Figure 6.