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Myeloid-specific Expression of Api6/AIM/Spα Induces Systemic Inflammation and Adenocarcinoma in the Lung

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Abstract

In order to study the functional role of apoptosis inhibition of myeloid lineage cells in tumor formation, apoptosis inhibitor 6 (Api6/AIM/Spα) was overexpressed in a myeloid-specific c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mouse model under the control of the c-fms promoter/intron 2. In this bitransgenic system, Api6-Flag fusion protein was expressed in myeloid lineage cells after doxycycline treatment. Induction of Api6 abnormally elevated levels of macrophages, neutrophils and dendritic cells in the bone marrow, blood and lung in vivo. BrdU incorporation and Annexin V binding studies showed systemically-increased cell proliferation and inhibition of apoptosis in myeloid lineage cells. Api6 overexpression activated oncogenic signaling pathways including Stat3, Erk1/2 and p38 in myeloid lineage cells in multiple organs of the bitransgenic mice. In the lung, severe inflammation and massive tissue remodeling were observed in association with increased-expression of pro-cancer cytokines/chemokines, decreased-expression of pro-apoptosis molecule genes and increased-expression of matrix metalloproteinase genes as a result of Api6 overexpression. Oncogenic CD11b+/Gr-1+ myeloid-derived suppressor cells (MDSCs) were systemically increased. After Api6 overexpression, lung adenocarcinoma was observed in bitransgenic mice with a 35% incidence rate. These studies suggest that dysregulation of myeloid cell populations by extracellular Api6 signaling leads to abnormal myelopoiesis and lung cancer.

Keywords

Myeloid lineage cells; Apoptosis; Inflammation; Lung cancer; Transgenic mice

Introduction

Inflammation is a major mechanism to protect organisms from damage in responding to pathogen infection and tissue injury. The process of inflammation involves interactions between migrating cells (e.g. macrophages, neutrophils, dendritic cells) and residential cells (e.g. epithelial cells, endothelial cells) in organs. This often orchestrated by secretion of extracellular signaling molecules from these cells in a paracrine fashion. Failure to clear excessive amounts of inflammatory cells, or to control aberrant expression of pro-inflammatory molecules at the inflammatory sites can cause further organ remodeling and damage, including cancer (1).

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The amounts of inflammatory cells in an organ depend on three factors: 1) cell infiltration; 2) cell proliferation and 3) programmed cell death (apoptosis). Apoptosis plays a key role in controlling tissue inflammation by limiting amounts of infiltrated immune cells. This is achieved by signal cascades triggered by extracellular stimuli in responding to physiological and environmental changes. Apoptosis can be induced through both intrinsic and extrinsic pathways (2). The extrinsic pathway is a receptor-mediated activation of initiator caspases that requires adaptors. The intrinsic pathway is initiated by damage to mitochondria or other organelles leading to activation of caspases. The balance between positive and negative apoptosis critically influences the inflammatory progression and tissue remodeling. Despite the identification of many intracellular apoptosis inhibitory elements, little is known about the negative (inhibitory) extracellular molecules that regulate apoptosis at the inflammatory sites.

Apoptosis inhibitor 6 (Api6/AIM/Spa) has been implicated to play a very important role in regulating immune cells. Api6 is a secreted protein from macrophages and belongs to the macrophage scavenger receptor cysteine-rich domain superfamily (SRCR-SF) (3,4). Gene ablation studies showed that Api6 plays a negative role in cell apoptosis at the inflammatory sites and functions as a modulator in immune response (4). Api6 inhibits apoptosis of monocytes, T cells, NKT cells and double positive thymocytes awaiting maturation in the thymus (4) (5). TGF-β and Api6 protein exposure blocks B-lymphocyte proliferation and Ig production (6). Api6 production facilitates macrophage survival within atherosclerotic lesions and loss of Api6 decreases early lesion development by increasing macrophage apoptosis (7). Api6 supports macrophage survival against apoptosis inducing stimuli in liver inflammatory lesions and enhances the phagocytic activity of macrophages (6). Api6 plays a role in macrophage survival in response to bacterial infection (8). Api6 has been shown to bind to the surface of Gram-positive and negative bacteria, which was mediated by recognition of lipoteichoic acid (LTA) and lipopolysaccharide (LPS) (9), suggesting its role in host defense. Taken together, Api6 is an immune regulator by inhibiting immune cell apoptosis.

We recently demonstrated that Api6 overexpression contributes to disease formation during lysosomal acid lipase (LAL) deficiency. LAL hydrolyzes cholesteryl ester and triglycerides to free cholesterol and free fatty acids in lysosomes. Inactivation of this neutral lipid metabolic enzyme leads to diseases in multiple organs (10–13). During LAL deficiency in LAL knock-out mice (*lal* −/−), Api6 was highly overexpressed (70 fold) in association with massive neutrophil influx and foamy macrophage accumulation in the lung, which caused “smoking-like” pulmonary remodeling (emphysema and hypercellularity) (11), suggesting that Api6 has more functions than just inhibiting apoptosis. Further characterization indicated that Api6 is a downstream target gene of LAL and lipid mediator peroxisome proliferator-activated receptor gamma (PPARγ)l (12). Since Api6 was originally identified in macrophages and Api6 overexpression was associated with macrophage accumulation and neutrophil infiltration in the *lal* −/− mice, we decided to specifically overexpress Api6 in myeloid lineage cells to evaluate its pathophysiological role in tissue inflammation and remodeling by taking the advantage of a monocyte/macrophage-specific doxycycline-inducible bitransgenic model system that was recently generated in our laboratory (14).

In this system, the “activator” transgenic mouse line bears the reverse tetracycline-responsive transactivator (rtTA) fusion protein under the control of the 7.2 kb 5’-flanking regulatory sequence and the downstream intron 2 of the c-fms gene (designated as c-fms-rtTA mice). The c-fms gene encodes the receptor for macrophage colony-stimulating factor (CSF-1) and is selectively expressed in macrophage lineages (15). Therefore, the rtTA expression is restricted to macrophages in transgenic mice (14). In the second transgenic mouse line, the Api6 gene is under the control of the tet operator DNA binding sequence that is linked to a minimal promoter (designated as $(TetO)_7$-CMV-Api6 mice). After crossbreeding, expression of the Api6 gene is induced by addition of doxycycline in bitransgenic mice (designated as c-fms-rtTA/$(TetO)_7$-

CMV-Api6 bitransgenic mice). Our results showed that induction of Api6 caused massive systemic increase of macrophages, neutrophils and dendritic cells (DCs) in multiple organs. Most strikingly, Api6 overexpression led to lung inflammation and formation of bronchoalveolar adenocarcinoma. Through these studies, two new features were revealed for Api6 in addition to apoptosis inhibition: 1) it increases infiltration of inflammatory cells into multiple organs; 2) it stimulates cell proliferation (both immune cells and epithelial cells) and tumor formation, acting like an oncogene. Oncogenic intracellular signaling pathways including Stat3, Erk1/2 and p38 were activated in both inflammatory cells and epithelial cells in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. In the lung, expression of pro-cancer cytokines/chemokines and tissue remodeling matrix metalloproteinase (MMPs) genes were significantly induced, whereas pro-apoptosis genes were significantly decreased.

Materials and Methods

Animal care

All scientific protocols involving the use of animals in this study have been approved by the Institution Animal Care and Usage Committee (IACUC) of Indiana University School of Medicine, and followed the guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association. Protocols involving the use of recombinant DNA or biohazard materials have been approved by the Institutional Biosafety Committee and followed the guidelines established by the NIH. Animals were housed under IACUC-approved conditions in a secured animal facility at Indiana University School of Medicine. Animals were regularly screened for common pathogens (specific pathogen free, or SPF). Experiments involving animal sacrifice utilize CO_2 narcosis to minimize animal discomfort.

Generation of doxycycline-controlled Api6 transgenic mice

To generate the $(TetO)_7$-CMV-Api6 transgenic mouse line, murine Api6 cDNA was amplified by PCR using a downstream primer (5'-AAGGAAAAAAGCGGCCGCTTATCACTTGTCATCGTCGTCCTTGTAGTCCACATC AAAGTCGTGGCA-3' with the Not I site and the Flag sequence) and an upstream primer (5'-CGCGGATCC GCCACCATGGCTCCATTGTTCAAC-3' with the Bam H1 site and the Kozak sequence). The PCR product was digested with BamH1/Not I and subcloned downstream of the CMV minimal promoter linked to seven Tet-responsive elements at the BamH1 and Not I sites in the pTRE2 vector (Clontech, Mountain View, CA). The expression cassette containing the CMV minimal promoter, the Api6 cDNA, and the human globin polyadenylation signaling sequence was dissected out and purified for microinjection into FVB/N mice. Founder lines were identified by a pair of primers corresponding to a pTRE2 plasmid sequence (5'-GCAGGATGATTACCAGGATGTAG-3') and an Api6 cDNA coding region sequence (5'-GGAGAAAAGGA GGACCAAGTGG-3'). The c-fms-rtTA transgenic mice were generated and genotyped as we previously reported (14). c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice were obtained by crossbreeding the c-fms-rtTA transgenic mice and $(TetO)_7$-CMV-Api6 transgenic mice. In general, animals were treated with doxycycline at 1 month-old age after genotyping.

FACS analysis

Bone marrow cells were flushed from femurs and resuspended in FACS buffer (PBS, 2% fetal bovine serum, 0.01% sodium azide). The spleen single-cell suspension was obtained by grinding and filtration through nylon mesh into FACS buffer. Blood mononuclear cells were obtained after red blood cell lysis with distilled water plus 10 X PBS. To prepare cells from the lung, the left atrium was opened by incision, and the right ventricle was infused with at least 2 ml of sterile PBS to remove any residual blood in the pulmonary vasculature. Lungs were cut into small pieces and placed in RPMI 1640 containing 5% FBS, collagenase (Sigma-

Aldrich), and DNase (Boehringer Mannheim). After 40 minutes of collagenase digestion at 37°C, lungs were further disrupted by aspiration through an 18-gauge needle and were suspended in FACS buffer. Approximately 1 to 2 × 10^6 cells from various organs in FACS buffer were blocked with FcR followed by incubation with primary antibodies. Cell surface markers Anti-CD11c (N148), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-CD3 (145-2C11) and anti-B220 (RA3-6B2) antibodies were purchased from eBiosciences (San Diego, CA). Cells were analyzed on LSRII machine (BD Biosciences, San Jose, CA). Data was analyzed using the BD FACStation™ Software (BD Biosciences). The total number of positive cells was calculated as the percentage of total gated viable cells. Isotype controls IgG1, IgG2a and IgG2b were included in all experiments. Quadrants were assigned using isotype control.

For measurement of intracellular signaling molecules, the assays were performed according to the protocols from Cell Signaling Technology (Danvers, MA) and a previously described procedure (16–19). Briefly, after surface staining, cell suspensions from bone marrow, blood, spleen and lung of doxycycline-treated or untreated c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice were fixed with 2% formaldehyde. Fixation samples were resuspended in methanol at a final 90% concentration. Finally, samples were washed and resuspended in 1 x PBS containing 4% fetal bovine serum (FBS) at 10^6 cells in 100 μl. Cell suspensions were labeled with the primary phospho-specific antibodies for 30 min at room temperature, then washed in PBS containing 4% FBS and labeled using the secondary antibody. After washing, samples were analyzed on a LSRII machine (BD Biosciences). Data was analyzed using the BD FACStation™ Software (BD Biosciences). Quadrants were assigned using isotype control. Anti-Phospho-P44/42 Mark antibody (T202/Y204, #4374), anti-Phospho-Stat3 antibody (Tyr 705, #4323) and anti-Phospho-P38 Mark antibody (Thr180/Tyr 182, #4551) were purchased from Cell Signaling Technology. SP-C antibody (FL-197) was from Santa Cruz Biotechnology. Monoclonal ANTI-FLAG® M2-FITCantibody (F4049) was from Sigma (Saint Louis, MI).

Alveolar type II epithelial cell purification

Alveolar type II epithelial cells were purified from Wild-type mice (WT), doxycycline-treated or untreated bitransgenic mice as described previously (12,20–22). After purification, alveolar type II epithelial cells were stained with anti-CD11b and Gr-1 antibodies to prove that no macrophages and neutrophils were contaminated.

Histology of lung

The lung from doxycycline-treated or untreated c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice was infused with a fixative solution (4% paraformaldehyde, 1 × phosphate-buffered saline) and was dissected out and stored in fixative at 4°C for ~24 h. After fixation and embedding in paraffin, lung tissue sections were cut to 5 μm thick. The adult lung slides were baked at 60°C for 2 h and washed in a series of xylene and ethanol to remove paraffin from the tissues. Multiple sections from each lung were stained with hematoxylin and eosin. Tumor incidence and multiplicity in each section was counted.

Real-Time PCR

All reverse transcription reactions were set up using the Taqman Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Two μg of total RNAs from the whole lungs, alveolar macrophages or alveolar type II epithelial cells of doxycycline-treated or untreated c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice were used in 100 μl of reaction mixture. The reactions were carried out using a GeneAmp 9700 Thermocycler (Applied Biosystems) with a suggested cycling protocol of 25°C for 10 mins, 48°C for 30 mins, and 95°C for 5 mins. For Real-Time PCR, 2 μl of cDNA was amplified by a pair of sequence-specific DNA oligonucleotide primers

for each molecule in a 50 μl of reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems). GAPDH primers were used as an endogenous control for normalizing all cDNA samples. The reactions were analyzed using the Relative Quantification Assay and 7500 System Sequence Detection Software for the 7500 Real-Time PCR System (Applied Biosystems).

Primers for Real-Time PCR:

mApaf-1

Upstream primer: 5'-CAG TAA TGG CGT CTT GTC AGT GA – 3'

Downstream primer: 5"-CGT TGA TAT TGA GTG GCC TGA CT- 3"

mBax

Upstream primer: 5'-GGG CCC ACC AGC TCT GA- 3'

Downstream primer: 5'-TGG ATG AAA CCC TGT AGC AAA A- 3'

mCasp3

Upstream primer: 5'-TGC TTA CTC TAC AGC ACC TGG TTA CT- 3'

Downstream primer: 5'-TGA ACC ACG ACC CGT CCT T- 3'

mCasp7

Upstream primer: 5'-CCG TCC ACA ATG ACT GCT CTT- 3'

Downstream primer: 5'-GGT CCT CCT CAG AGG CTT TTC- 3'

mCasp9

Upstream primer: 5'-AAC GAC CTG ACT GCC AAG AAA- 3'

Downstream primer: 5'-GGT TCC GGT GTG CCA TCT C- 3'

mCideA

Upstream primer: 5'-CAGCAGCCTGCAGGAACTTAT- 3'

Downstream primer: 5'-ACCAGGCCAGTTGTGATGACT- 3'

mCcl5

Upstream: 5'-GGAGTATTTCTACACCAGCAGCAA-3'

Downstream: 5'-CGGTTCCTTCGAGTGACAAAC -3'

mCcl8

Upstream: 5'-AAAGCTACGAGAGAATCAACAATATCC -3'

Downstream: 5'-CCTGCTTGGTCTGGAAAACC-3'

mCxcr2

Upstream: 5’-CTGCTCACAAACAGCGTCGTA -3’

Downstream: 5’-CTTGAATTCTCCCATCTTTGAGGTA-3’

mGp130

Upstream: 5’-CCCATGGGCAGGAATATAGATC-3’

Downstream: 5’-TTCCCATTGGCTTCAGAAAGA-3’

mLif

Upstream: 5’-GAGTCCAGCCCATAATGAAGGT-3’

Downstream: 5’-GTGCAGAACCAGCAGCAGTAAG -3’

mIL6

Upstream: 5’-GAGGCTTAATTACACATGTTC-3’

Downstream: 5’-TGCCATTGCACAACTCTTTTCT-3’

mIL1β

Upstream: 5’-TTGACGGACCCCAAAAGATG- 3’

Downstream: 5’-CAGGACAGCCCAGGTCAAA- 3’

mTnfsf9

Upstream: 5’-CGCCAAGCTACTGGCTAAAAA-3’

Downstream: 5’-GGCTGTGCCAGTTCAGAGTTG -3’

mVEGF

Upstream: 5’-CCCACGTCAGAGAGCAACATC-3’

Downstream: 5’-TGGCTTTGGTGAGGTTTGATC-3’

mCsf2

Upstream: 5’-CCTTGAACATGACAGCCAGCTA-3’

Downstream: 5’-CACAGTCCGTTTCCGGAGTT -3’

mMMP-2

Upstream: 5’-GAGGCTGACATCATGATCAACTTT- 3’

Downstream: 5’-GCCATCAAATGGGTATCCATCT- 3’

mMMP-7

Upstream: 5’-TGAGGACGCAGGAGTGAACTT- 3’

Downstream: 5’-CCCAGAGAGTGGCCAAATTC- 3’

mMMP-8

Upstream: 5’-CTCAAGAGACCATGGTGACAATTC- 3’

Downstream: 5’-AAGGCATGGGCAAGGATTC - 3’

mMMP-9

Upstream: 5’-GAGGCTGACATCATGATCAACTTT- 3’

Downstream: 5’-GCCATCAAATGGGTATCCATCT- 3’

mMMP-12

Upstream: 5’-TGGTATTCAAGGAGATGCACATTT- 3’

Downstream: 5’-GGTTTGTGCCTTGAAAACTTTTAGT- 3’

Apoptosis assay

Dual staining with fluorescein isothiocyanate-labeled-annexin V (FITC-annexinV) and propidium iodide (PI) was performed to detect cells undergoing apoptosis by using an Annexin V-FITC kit (BD Biosciences, Bedford, MA). Briefly, cells from different tissues of doxycycline-treated or untreated c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice were isolated as mentions above. After staining with cell surface-specific markers (e.g. CD11b, CD11c, Gr-1, CD3, B220), cells were washed twice with PBS. After resuspension of labeled cells in annexin V-binding buffer containing FITC-conjugated annexin V, PI was added into samples for 10 min incubation on ice. Cells were analyzed on a LSRII machine within one hour. At least 1×10^4 cells in each sample were analyzed. Control cells stained with annexin V-FITC or PI alone were used to compensate for the flow cytometric analysis. Annexin V and PI double-negative cells are defined as live cells. Annexin V-positive, PI-negative cells are defined as early apoptotic cells. Annexin V and PI double-positive cells are defined as late apoptotic and necrotic cells. Nonspecific binding was blocked by pre-incubating the cells with rat IgG (10 μg/ml) and anti-FcII/III.

Cell proliferation

For the cell proliferation study, littermates of doxycycline-treated or untreated c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice were inoculated i.p. with 1 mg BrdU/animal (Sigma-Aldrich) in PBS for 24 hrs. Cells from different tissues were harvested and stained with surface markers (e.g. CD11b, CD11c, Gr-1, CD3, B220) as mentioned above. Labeled cells were washed twice with PBS and treated with DNase I followed by staining with fluorescein–anti-BrdU using BrdU flow kit (BD BioSciences) before being analyzed on a LSR II machine.

Bone-marrow derived macrophages

For *in vitro* assay, bone marrow-derived macrophages were obtained as described previously (23). Briefly, femurs from wild-type mice were flushed using a 26-gauge needle and bone marrow cells were cultured for 3 hours. Nonadherent cells were removed and cultured in the minimum essential medium α supplemented with 10% fetal calf serum and 50 μm of 2-mercaptoethanol and 10 ng/mL of recombinant murine IL-3 and 10 ng/mL of recombinant murine GM-CSF for 14 days. As soon as the monocytes started to adhere (6–7 days), all nonadherent cells were washed away from the culture to minimize potential interactions with other cells. More than 90% of the bone marrow population was F4/80+ macrophages as determined by flow cytometry.

In vitro function analysis of Api6

Macrophages isolated above were cultured in RPMI -1640 culture medium with or without 100 ng/ml of Api6 (R&D company, Minneapolis, MN, USA) or 1 μg/ml of LPS for 24 hours. BrdU (10 μm, final concentration) was added to cells at the last hour. Cell proliferation was characterized by using the BrdU flow kit and F4/80 antibody (17–4801–82, eBioscience). *In vitro* apoptotic assay of Api6 was characterized by using Annexin V flow kit and F4/80 antibody.

Statistical analysis

The data shown were mean values of at least three independent experiments and expressed as Mean±SD. A paired Student's t test or ANOVA was used to evaluate the significance of the differences. Statistical significance was set at a level of $P< 0.05$. Survival probability was calculated according to the Kaplan-Meier Method. Differences in survival were assessed with the log-rank test.

Results

Generation of c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice

A doxycycline-controlled bitransgenic mouse model was generated to specifically direct Api6 expression in myeloid cells. Supplemental Figure 1A illustrates myeloid lineage specificity of the c-fms promoter/intron2. In this system, a previously established c-fms-rtTA transgenic mouse line (14) was crossbred with a newly generated $(TetO)_7$-CMV-Api6 transgenic mouse line. Bitransgenic mice were obtained as detected by PCR using specific primers and mouse tail DNA (Supplemental Figure 1B). In order to assess the expression pattern of Api6-Flag fusion protein in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice, a Flag sequence was added at the C terminus of the Api6 cDNA to distinguish exogenous Api6-Flag fusion protein from endogenous Api6 protein. Bitransgenic mice were treated with or without doxycycline for 4 months and were sacrificed for FACS analysis. Single-cell suspensions from the bone marrow, blood, spleen and lung were double stained with fluorochrome-conjugated Flag antibody and antibodies specific for macrophages, dendritic cells (DCs), neutrophils, or T cells. CD11b+ macrophages, Gr-1+ neutraphils and CD11c+ DCs showed Api6-Flag overexpression in all tested organs of doxycycline-treated mice vs untreated mice (Figure 1A&B). As a negative control, there was no Api6-Flag induction in CD3+ T lymphocytes regardless of doxycycline treatment. These results demonstrated that Api6-Flag overexpression in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice is myeloid cell specific, rather than monocyte/macrophage specific as originally reported (15). No Api6-Flag fusion protein was detected in wild type mice regardless of doxycycline treatment (data not shown), suggesting that induction of Api6-Flag fusion protein was not caused by doxycycline alone.

Overexpression of Api6 caused systemic increases of macrophages, DCs and neutrophils

To assess the pathogenic effect of overexprssion of Api6 on immune cells, bitransgenic mice were treated with or without doxycycline for 3 months. The cells from the bone marrow, blood, spleen and lung were isolated and stained with fluorochrome-conjugated antibodies specific for macrophages, DC, neutrophils, B cells or T cells for FACS analysis. Compared to doxycycline-untreated mice, the number of CD11b+ macrophages were increased from 54.77% to 67.87% in the bone marrow, 5.96% to 22.82% in the blood (PBMC), 1.91% to 4.22% in the spleen and 10.92% to 21.06% in the lung of doxycycline-treated bitransgenic mice (Figure 2A). In wild type mice control, no change was observed regardless of doxycycline treatment (data not shown). As a result, the total cellular numbers of the bone marrow, spleen and lung in treated mice were modestly increased compared to those in doxycycline-untreated mice and wild type mice (Figure 2B). The spleen weight was modestly increased in

doxycycline-treated bitransgenic mice compared to untreated mice (Figure 2C). Neutrophils also showed increases in the blood, spleen and lung (data not shown). In contrary, no increase of T and B lymphocytes was observed in bitransgenic mice regardless of doxycycline treatment (data not shown). Therefore, increase of Api6 expression in myeloid cells can increase systemic myeloid cell numbers in various organs of bitransgenic mice.

Overexpression of Api6 inhibited apoptosis of macrophages, neutrophils and DCs

To determine whether increased macrophages, neutrophils and DCs were a result of apoptosis inhibition by Api6 overexpression, direct comparison of cellular apoptosis by annexin V analysis was made *in vivo*. Single cells were purified from the bone marrow, blood, spleen and lung of 3-month doxycycline-treated, untreated bitransgenic mice and wild type mice. They were co-stained with fluorochrome-conjugated annexin V antibody and cell surface-specific antibodies for macrophages, DC, neutrophils, B cells or T cells. In CD11b+ (Figure 3A) and Gr-1+ cell populations (Figure 3B), significant inhibition of apoptosis was observed in all tissues, compared to those from doxycycline-untreated mice and wild type mice, especially in the lung. In the CD11c+ DC population, inhibition of apoptosis was observed in the same samples except that in the bone marrow and the lung (Figure 3C). No change of apoptosis was observed in the CD3+ T lymphocyte and B220+ B lymphocyte populations (data not shown). These observations indicate that overexpression of Api6 increases survival of myeloid cells at various maturation stages in various organs by apoptosis inhibition. This was further supported by an *in vitro* experiment, in which addition of Api6 protein inhibited cultured macrophage apoptosis (Figure 3D and E).

Overexpression of Api6 induced proliferation of macrophages, neutrophils and DCs

Increased numbers of macrophages, neutrophils and DCs in Api6 bitransgenic mice can also be caused by the increased activity of cell proliferation. This has never been tested before for Api6. To test this possibility, BrdU labeling analysis was performed. Doxcycline treated (3-month) or untreated bitransgenic mice received i.p. injections of BrdU for 24 hrs and were sacrificed on the next day. In the same cell populations outlined above, single cells were co-stained with fluorochrome-conjugated BrdU antibody and cell surface-specific antibodies for macrophages, DCs, neutrophils, B cells or T cells. Api6 overexpression modestly increased cell proliferation of CD11b+ and GR-1+ cells in the bone marrow, blood and lung, except for the spleen, compared to those in doxycycline-untreated mice and wild type mice (Figure 4 A-B). There was no statistical difference for the percentages of proliferating CD11c+ DCs (Figure 4C) and CD3+ T lymphocytes and B220+ B lymphocytes in bitransgenic mice (data not shown). These studies demonstrated that Api6 is able to stimulate cell proliferation of most myeloid lineage cells in multiple organs. Again, this was further supported by an *in vitro* experiment, in which addition of Api6 protein stimulated proliferation of cultured macrophages (Figure 4D and E).

Activation of intracellular signaling molecules by Api6 overexpression

The intracellular signaling molecules that mediate the Api6 action have never been identified. To identify intracellular signaling pathways that are activated by Api6 overexpression in myeloid cells, activation of Stat3, Erk1/2, Akt, p38 and NFκB p65 phosphorylation was tested. In the same cell populations outlined above, single cells from the bone marrow, blood and lung from 3-month doxycycline-treated or untreated bitransgenic mice were co-stained with fluorochrome-conjugated anti-phosphor-Stat3, anti-phosphor-Erk1/2, anti-phosphor-Akt or anti-phosphor-p38 antibody and cell surface-specific antibodies for macrophages, DCs and neutrophils. FACS showed increased numbers of phosphor-Stat3, phosphor-Erk1/2 and phosphor-p38 positive cells in CD11b+ macrophages, CD11c+ DCs and GR-1+ neutrophils of doxycycline-treated bitransgenic mice (Figure 5 and Table 1). The only exception is that no

change on Stat3 phosphorylation was observed in above myeloid cells in the bone marrow regardless of doxycycline treatment (Table 1). Changes of Akt and NFκB p65 phosphorylation were observed in none of these immune cells (data not shown). Mean fluorescence intensity (MFI) by FACS indicated that the signaling intensities of phosphor-Stat3, phosphor-Erk1/2, and phosphor-p38 were significantly increased in blood and lung CD11b+ macrophages and GR-1+ neutrophils. Changes with statistical significance were not observed in the bone marrow (Table 2). Therefore, activation of multiple intracellular signaling pathways in myeloid cells by Api6 implicates that Api6 is a multifunctional extracellular signaling molecule that has diverse biological functions in multiple organs.

Overexpression of Api6 in myeloid cells induced bronchoalveolar adenocarcinoma in the bitransgenic lungs

Recently, we demonstrated that there is a tight connection between chronic inflammation and lung cancer formation (21). Since Api6 overexpression caused pulmonary inflammation, pathogenesis of doxycycline-treated or untreated c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice were analyzed by histology. Severe lung inflammation and tissue remodeling with hypercellularity were observed in 3-month doxycycline-treated bitransgenic mice (data not shown). With 6 to 11-month induction of Api6, multiple animals (14 out of 40, or 35%) developed bronchoalveolar adenocarcinoma in the bitransgenic lungs (Figure 6A & B). No tumor was observed in the lung of doxycycline-untreated control mice. In a survival study, more than 50% of bitransgenic mice were died within 8-month doxycycline treatment (n =30), compared to 10% in untreated bitransgenic mice (n =28) and wild type mice (n =35) (Figure 6C). The differences of death rates between treated and untreated Api6 bitransgenic mice were statistically significant based on the statistical analysis (the Kaplan-Meier Method, differences were assessed with the log-rank test). These results strongly support that Api6 is an oncogene that induces lung bronchioalveolar adenocarcinoma by promoting inflammation.

Overexpression of Api6 in myeloid cells induced oncogenic microenvironment change in the bitransgenic lung

Persistent activation of the pro-inflammatory Stat3 pathway in lung alveolar type II epithelial cells directly caused bronchioalveolar adenocarcinoma (21). Since Api6 is a secretory protein and its expression was detected in the lung (Figure 1B), it is possible that Api6 induces lung bronchioalveolar adenocarcinoma through stimulating the IL6/Stat3 oncogenic pathway in lung epithelial cells. Indeed, expression of IL-6 and Stat3 was highly induced in the lung (including alveolar type II epithelial cells and alveolar macrophages) of doxycycline-treated bitransgenic mice compared to untreated or wild type mice (Figure 7A and B). The isolated alveolar type II epithelial cells contained less than 0.3% of CD11b and Gr-1 positive cells (Supplemental Figure 2A). In addition, numbers of alveolar type II epithelial cells with the active Stat3 (phosphor-Stat3) were significantly increased (Figure 7C). A group of Stat3 downstream pro-cancer cytokines and chemokines have been identified as we recently reported (21). All these molecules were highly induced in alveolar type II epithelial cells and alveolar macrophages of doxycycline-treated bitransgenic mice compared to untreated mice (Figure 7D). The macrophage purity was more than 97% (Supplemental Figure 2C). Tumor requires a constant influx of myelomonocytic cells to support the angiogenesis and stroma remodeling for their growth. One such cell population is myeloid-derived suppressor cells (MDSCs) commonly expressing both Gr-1 and CD11b in mice (24). Three months after Api6 induction in bitransgenic mice, the percentage number of Gr-1+CD11b+ cell population was drastically increased in the blood (from 2.11% to 16.50%), spleen (from 1.27% to 6.33%) and lung (from 4.79% to 31.69%) compared to untreated bitransgenic mice (Figure 8A). The total number of this cell population was gradually increased in the lung of doxycycline-treated bitransgenic mice (Figure 8B).

Api6 overexpression altered expression levels of apoptotic and MMP genes in bitransgenic lungs

Tumor formation is a complex process that involves multiple steps and malfunction of many genes. To test pathogenic gene expression associated with tumorigenesis in Api6 bitransgenic lungs, we first examined the expression levels of a spectrum of apoptotic genes (Apaf-1, Bax, Bcl-2, Bid, Casp3, 7, 9, CideA, FasL). Total RNAs were isolated from whole lungs, alveolar type II epithelial cells and alveolar macrophages of 3 month doxycycline-treated or untreated bitransgenic mice for RNA purification. By Real-Time PCR analysis, expression levels of pro-apoptotic molecules were all significantly reduced in bitransgenic lungs, alveolar type II epithelial cells and alveolar macrophages after doxycycline treatment (Figure 9A). In contrast, the expression level of the anti-apoptosis Bcl-2 gene was increased. MMPs are major tissue remodeling enzymes that support tumor formation and progression. In doxycycline-treated bitransgenic lungs, mRNA expression levels of MMP-2, 7, 8, 9 and 12 were highly induced in alveolar type II epithelial cells and alveolar macrophages in responding to Api6 overexpression in myeloid cells (Figure 9B), suggesting a robust tissue remodeling activity required for tumorigenesis. The above genes are potential downstream target genes for the Api6 extracellular signaling pathway. In wild type mice, no changes were observed in above molecules regardless of doxycycline treatment (data not shown), indicating that changes of gene expression were not caused by doxycycline.

Discussion

Myeloid lineage cells (e.g. macrophages, neutrolphils and DCs) are highly versatile cells and actively participate in tissue inflammation and remodeling by producing and secreting extracellular signaling molecules that influence gene expression, cell proliferation/differentiation and apoptosis in organ tissues through the paracrine mechanism. Although these cells regulate tumor growth, angiogenesis, invasion, and metastasis (24–26), there is no evidence indicating that malformation and malfunction of myeloid cells directly induce tumor formation in organs. Macrophages and neutrophils with malfunction (e.g. MDSCs) in the immune system or the hematopoietic system can enter organs. Abnormal secretion of oncogenic molecules from invading macrophages and neutrophils are able to change the local microenvironment by secreting pro-tumor cytokines/chemokines and transform local cells (e. g. epithelial or endothelial cells) into cancerous cells. This mechanism has not been vigorously tested due to lack of proper tools to specifically express “genes of interest” in myeloid lineage cell lines. This technical difficulty has been overcome by a myeloid-specific doxycycline controllable system that was established in our laboratories (14). This rather unique system allows us to systematically manipulate myeloid lineage cells by expressing “genes of interest” that contribute to tumor formation in various organs.

The first molecule we tested for expressing “genes of interest” in myeloid cells is Api6. This is based on our previous investigation that Api6 overexpression was highly associated with inflammation and tissue remodeling during deficiency of neutral lipid metabolism in vivo (12,14). Api6 has been demonstrated to be an immune regulator by controlling apoptosis of immune cells. After generation of the c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mouse model, expression of Api6-Flag fusion protein was systematically evaluated. The c-fms promoter/intron 2 was originally described as monocyte/macrophage specific (15). But in our assessment, this DNA sequence also rendered Gr-1+ neutrophil and CD11c+ DC expression of Api6-Flag fusion protein in various organs (Figure 1). Therefore, this is a myeloid cell-specific animal model rather than a monocyte/macrophage-specific animal model. It is not totally unexpected to see Api6 expression in Gr-1+ neutrophils of bitransgenic mice in the bone marrow, as macrophages and granulocytes share common progenitor cells, which is dependent on CSF-1 (15,27). However, mature granulocytes in other organs do not express the CSF-1

receptor. One explanation is that the c-fms promoter/intron 2 DNA sequence has a broader cell specificity than that previously proposed (15) due to removal of some critical DNA elements that determine cell lineage specificity. Api6-Flag fusion protein was not expressed in lymphoid lineage B and T lymphocytes as determined by flow cytometry analysis.

Overexpression of Api6 in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice by doxycycline-treatment enabled systemic increase of macrophages, neutrophils and dendritic cells in adult mice from the hematopoietic system (e.g. bone marrow and blood) to the immune system (e.g. spleen) to tissue organs (e.g. lung) (Figure 2A). Programmed cell death and proliferation are two factors that determine a given cell population in organs. Api6 plays a dual function to systemically increase numbers of myeloid cells in Api6 bitransgenic mice. Annexin V analysis showed the Api6 apoptotic inhibitory function (Figure 3A) while BrdU labeling analysis showed the Api6 cell proliferation stimulatory function (Figure 4A) on myeloid cells with a few exceptions *in vivo*. This observation has been confirmed by *in vitro* macrophage culturing experiments, in which Api6 protein stimulated cell proliferation and inhibited apoptosis of bone marrow-derived macrophages in the *in vitro* culturing condition (Figure 3A & 4A). In different organ systems, various myeloid cell types showed distinct apoptotic and proliferative patterns, reflecting contributions from local microenvironment. In order to understand the molecular mechanisms that underline Api6 induced apoptosis inhibition and cell proliferation, various important intracellular signaling molecules that are known to regulate apoptosis and cell proliferation were investigated, including Stat3, Erk, Akt, p38 and NFκB. In these studies, overexpression of Api6 can activate the Stat3 pathway and the Ras downstream Erk/p38 pathways as determined by intranuclear staining and FACS analysis (Figure 5 and Table 1&2). The activation of Stat3, Erk/p38 seemed more prominent in the peripheral blood and lung than that in the bone marrow. Both pathways are known to promote cell growth and inhibit apoptosis. The observation was reflected at both the increased levels of positively-labeled cell numbers and overall MFI signaling intensities. It is likely that Api6 stimulates cell proliferation by activating these pathways. In contrast, phosphorylation of Akt and NFκB p65 remained relatively unchanged.

Increased infiltration and accumulation of inflammatory cells that were caused by Api6 overexpression indeed changed the lung microenvironment at both cellular and molecular levels. The percentage number of Gr-1+CD11b+ MDSCs was systemically increased in Api6 bitransgenic mice, including the blood, spleen and lung (Figure 8). Expression of pro-tumor IL-6/Stat3 molecules and their downstream cytokines and chemokines were significantly elevated in the lung of Api6 bitransgenic mice, including macrophages and epithelial cells (Figure 7). These changes inevitably exert pathophysiological influences on residential cells (e.g. epithelial cells). In doxycycline-treated Api6 bitransgenic mice, epithelial cell outgrowth led to formation of bronchoalveolar adenocarcinomas with a 35% incidence rate (Figure 6). This new finding suggests that Api6 has the oncogenic function. The Api6 bitransgenic system showed a connection between chronic inflammation and tumor formation in the lung. It has been reported previously that persistent inflammation caused by Stat3 and Ras/mitogen-activated protein kinase (MARK) pathways resulted in formation of bronchoalveolar adenocarcinoma (21,28). Both pathways can be activated by Api6 overexpression. Other microenvironment changes in the Api6 bitransgenic lung include down-regulation of pro-apoptosis molecules (Apaf-1, Bax, Casp3, 7, 9 and CideA) and up-regulation of anti-apoptosis molecule (Bcl-2) (Figure 9A). This reassures the anti-apoptotic feature of Api6. Api6 overexpression also up-regulated MMPs in the lung (Figure 9B). These are critical extracellular matrix (ECM) degrading enzymes for tissue remodeling and cancer development (29). Both myeloid cells and epithelial cells can synthesize MMPs in responding to various inflammatory conditions as previously demonstrated (11,12,30,31).

In summary, we demonstrated for the first time that malformation and malfunction of myeloid-derived cells can cause lung cancer in the unique myeloid-specific Api6 bitransgenic system. Although Api6 was tested in this report, systematical evaluation of other secretory inflammatory molecules (cytokines, chemokines, growth factors) by using the same doxycycline-inducible system can reveal relationship between myeloid cells and tumor formation in various organs. In this report, two new functions of Api6 have been identified besides its anti-apoptosis function. First, Api6 has the oncogenic effect. It stimulates lung cancer formation originated from epithelial cell outgrowth in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic lungs. This has been confirmed by another animal model system (CCSP-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice) recently generated in our laboratory. In this system, Api6 overexpressed in lung epithelial cells directly caused formation of bronchoalveolar adenocarcinomas (data not shown). Since Api6 is a secretory protein, overexpression of Api6 in the lung from different cell sources (e.g. macrophages and epithelial cells) can generate the similar clinical outcomes (e.g. cancer). Both c-fms-rtTA/$(TetO)_7$-CMV-Api6 and CCSP-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic animal systems strongly support that Api6 is a potent oncogene. Second, Api6 is a pro-inflammatory molecule. It inhibits apoptosis and stimulates proliferation of myeloid cells in almost all tested organs of c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. Taken together, Api6 is a pleiotrophic molecule that induces organ inflammation and tumorigenesis. Studies outlined here once again provide strong evidence that persistent inflammation induces cancer formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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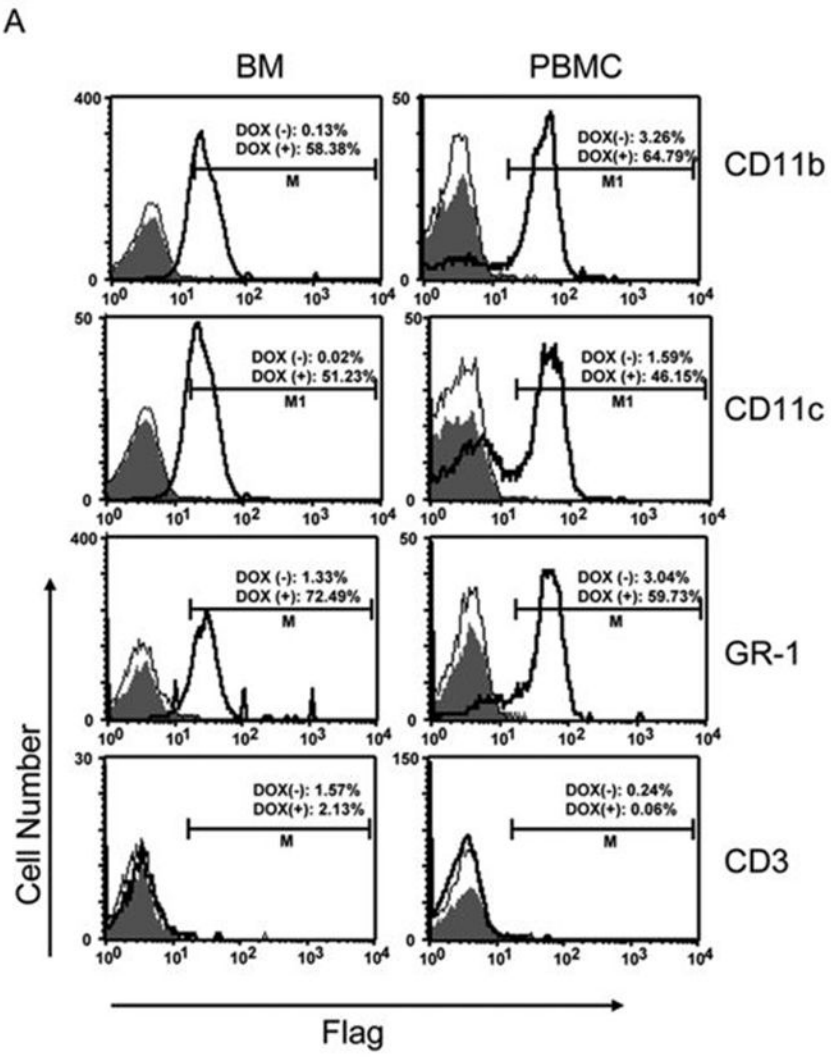


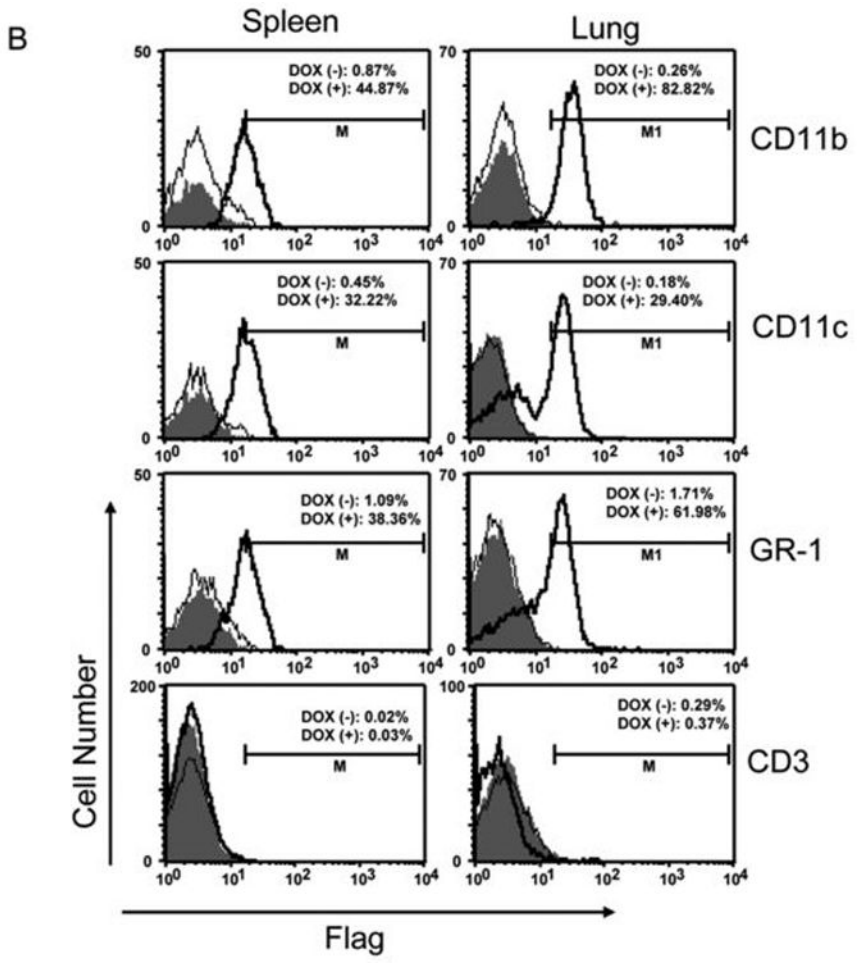
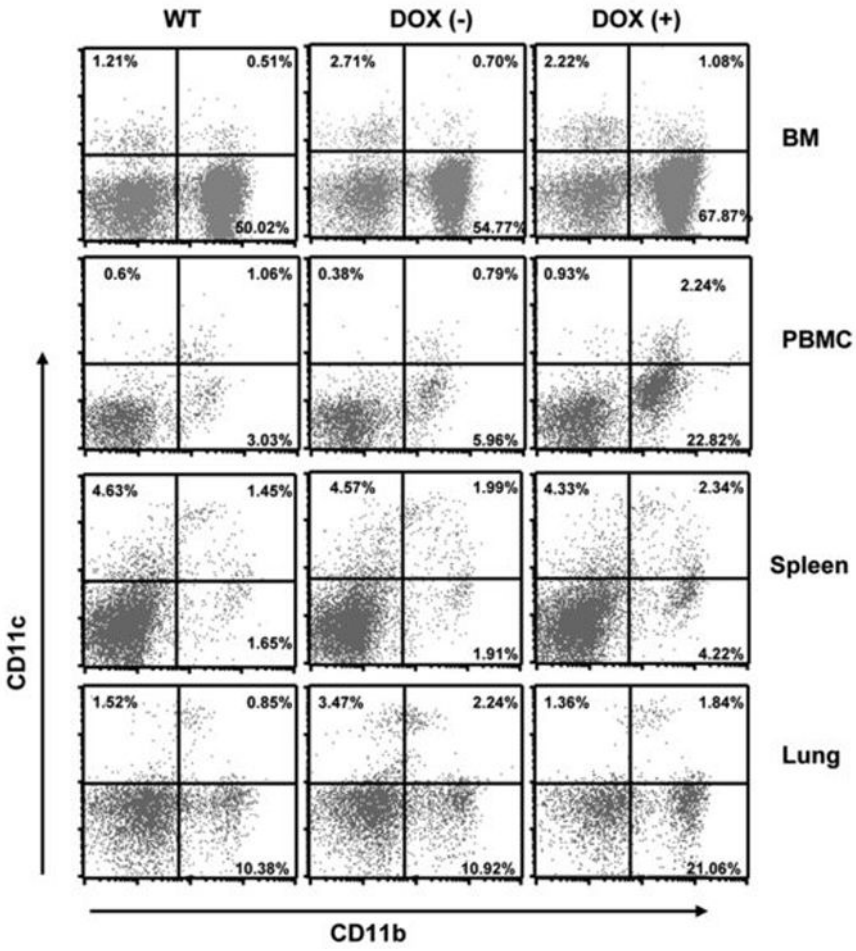


Figure 1.

Doxycycline-controllable c-fms-rtTA/(TetO)$_7$-CMV-Api6 bitransgenic mice showed myeloid specificity. Cells from the bone marrow, blood (PBMC), spleen and lung of 3-month doxycycline-treated [DOX(+), black line] or untreated [DOX(−), gray line] bitransgenic mice were double stained with anti-Flag antibody in combination with cell-surface markers CD11b, CD11c, GR-1 and CD3. In gated CD11b+, CD11c+, GR-1+ and CD3+ cells, numbers of the Flag+ cells were analyzed by flow cytometry in histograms. Isotype controls are shown as the shaded areas in each assay.



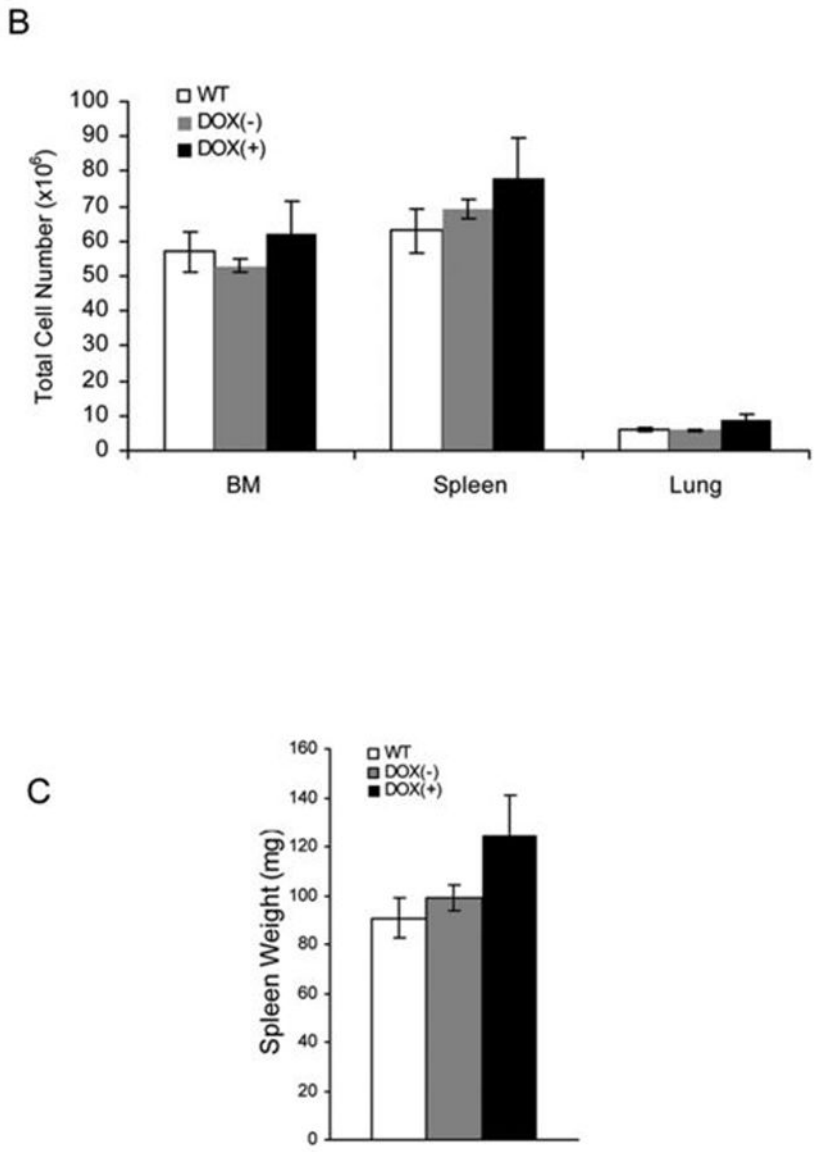
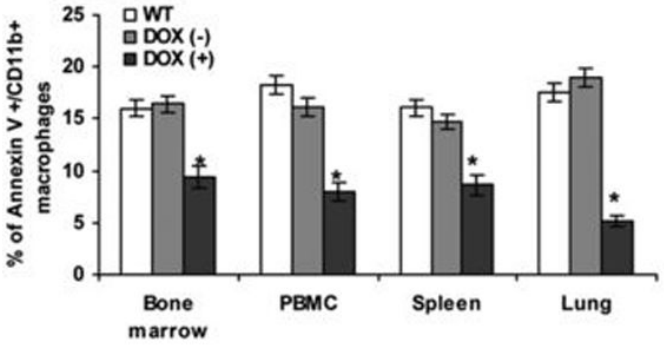


Figure 2.

Myeloid cells were systemically increased in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. **A)** Single cell suspensions were prepared from the bone marrow, blood (PBMC), spleen and lung of 3-month doxycycline-treated or untreated bitransgenic mice for FACS analyses. Distribution of CD11b+ macrophage and CD11c+ DCs is presented in dot plots. The analyses were repeated three times and a representative experiment is shown. Isotype control is illustrated in supplemental Figure 3. WT, wild type mice; DOX (−), doxycycline-untreated bitransgenic mice; DOX(+), doxycycline-treated bitransgenic mice. **B)** In the above samples, total cellularity was determined by using a hemocytometer and trypan blue exclusion of dead cells. **C)** The spleens from wild type, doxycycline-treated and untreated bitransgenic mice were weighed at 3 months old.

A



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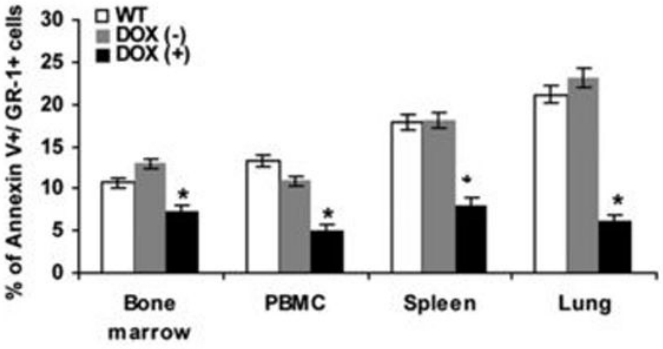

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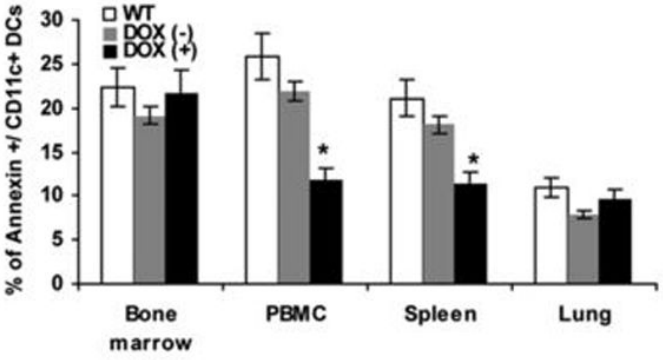


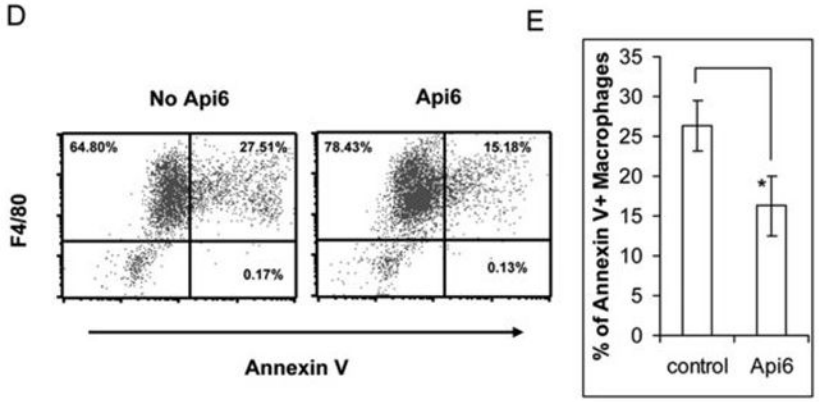
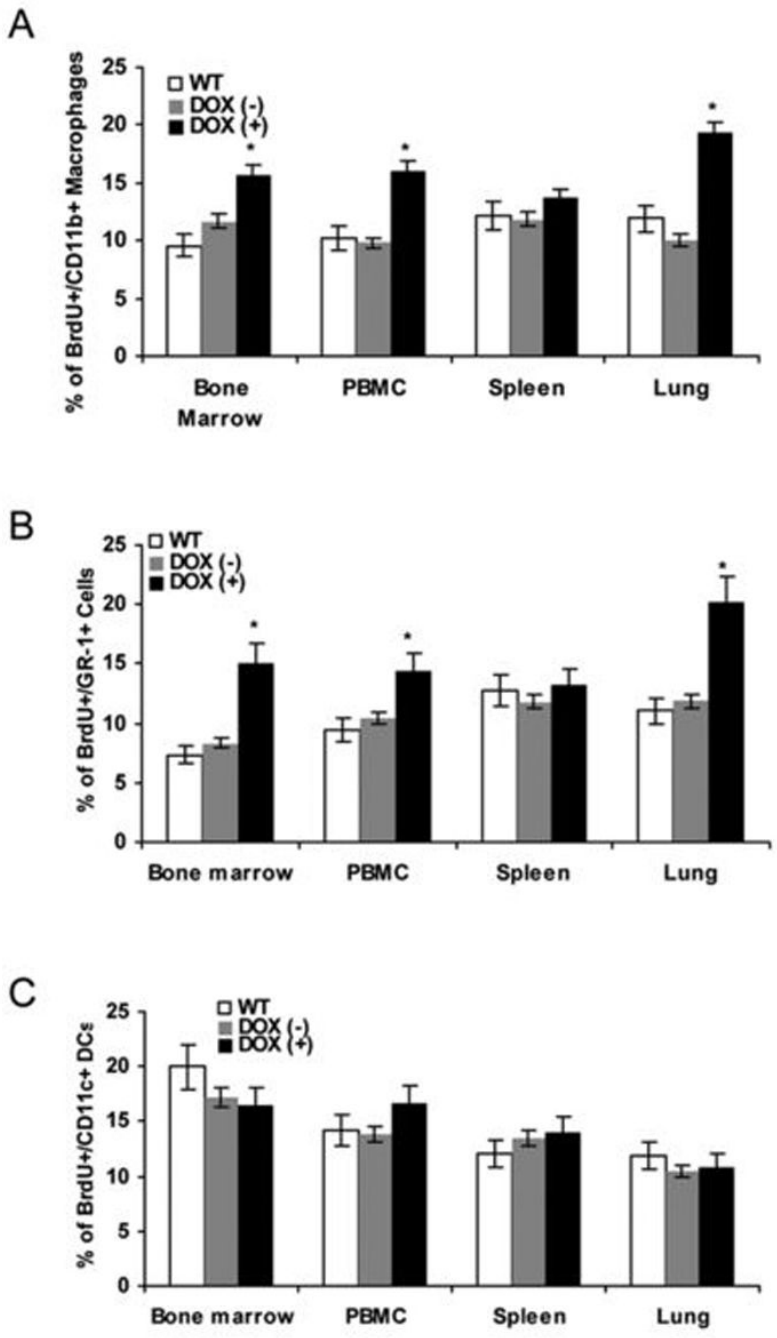


Figure 3.

Overexpression of Api6 inhibited apoptosis of myeloid lineage cells in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. **A-C)** In *in vivo* characterization, single cell suspensions were prepared from the bone marrow, blood (PBMC), spleen and lung of 3-month doxycycline-treated, untreated bitransgenic mice and age-matched wild type mice for FACS analyses. Relative apoptosis levels of CD11b+ macrophages (A), Gr-1+ neutrophils (B) and CD11c+ dendritic cells (C) were analyzed by annexin V. Data are presented as the means of three independent FASC experiments. *P< 0.05. WT, wild type mice; DOX (−), doxycycline-untreated bitransgenic mice; DOX(+), doxycycline-treated bitransgenic mice. **D-E)** In *in vitro* characterization, bone marrow-derived macrophages were cultured *in vitro* with or without 100 ng/ml of Api6 for 24 hours. Cell apoptosis was analyzed by Annexin V in histogram (D). Isotype control is illustrated in supplemental Figure 4. Data are presented as the means of three independent FASC experiments. *P< 0.05 (E).



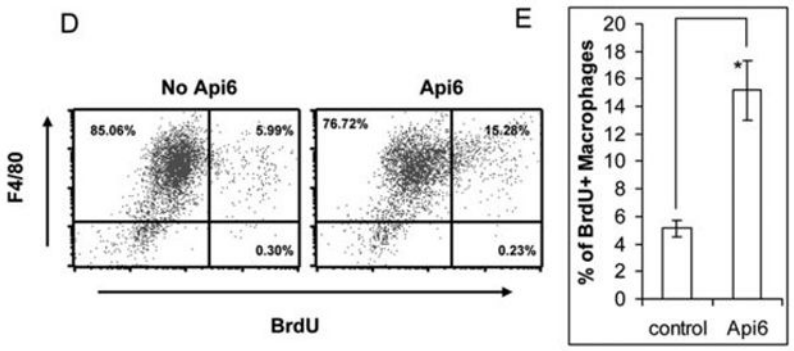


Figure 4.

Overexpression of Api6 stimulated proliferation of myeloid lineage cells in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. **A-C)** In *in vivo* characterization, after BrdU injection, single cell suspensions were prepared from the bone marrow, blood (PBMC), spleen and lung of 3-month doxycycline-treated or untreated bitransgenic mice and age-matched wild type mice for FACS analysis. Relative BrdU labeling levels of CD11b+ macrophages (A), Gr-1+ neutrophils (B) and CD11c+ DCs (C) were analyzed by BrdU in three independent FASC. Data are means of 4 independent experiments ±SD. *P<0.05. WT, wild type mice; DOX (−), doxycycline-untreated bitransgenic mice; DOX(+), doxycycline-treated bitransgenic mice. **D-E)** In *in vitro* characterization, bone marrow-derived macrophages were cultured *in vitro* with or without 100 ng/ml of Api6 for 24 hours. BrdU (10μm final concentration) was added to cells in the last hour. Cell proliferation was analyzed by BrdU in histogram (D). Isotype control is illustrated in supplemental Figure 5. Data are presented as the means of three independent FASC experiments. *, P< 0.05 (E).

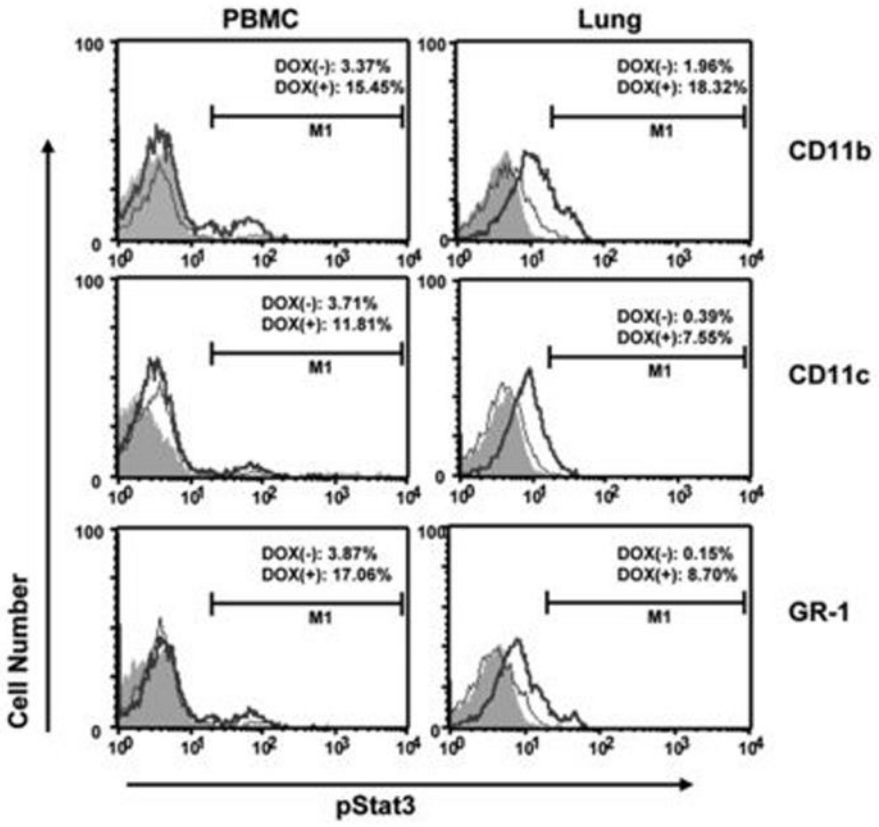


Figure 5.

Overexpression of Api6 activated Stat3 in myeloid lineage cells of c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. Single cell suspensions were prepared from the blood (PBMC) and lung of 3-month doxycycline-treated or untreated bitransgenic mice for FACS analyses of relative phosphor-Stat3 cell populations (pStat3) in myeloid cells. Each data represents 3 independent (n=3) studies of FACS analyses. DOX (−), doxycycline-untreated bitransgenic mice (gray line); DOX(+), doxycycline-treated bitransgenic mice (black line); Shaded areas are isotype controls.

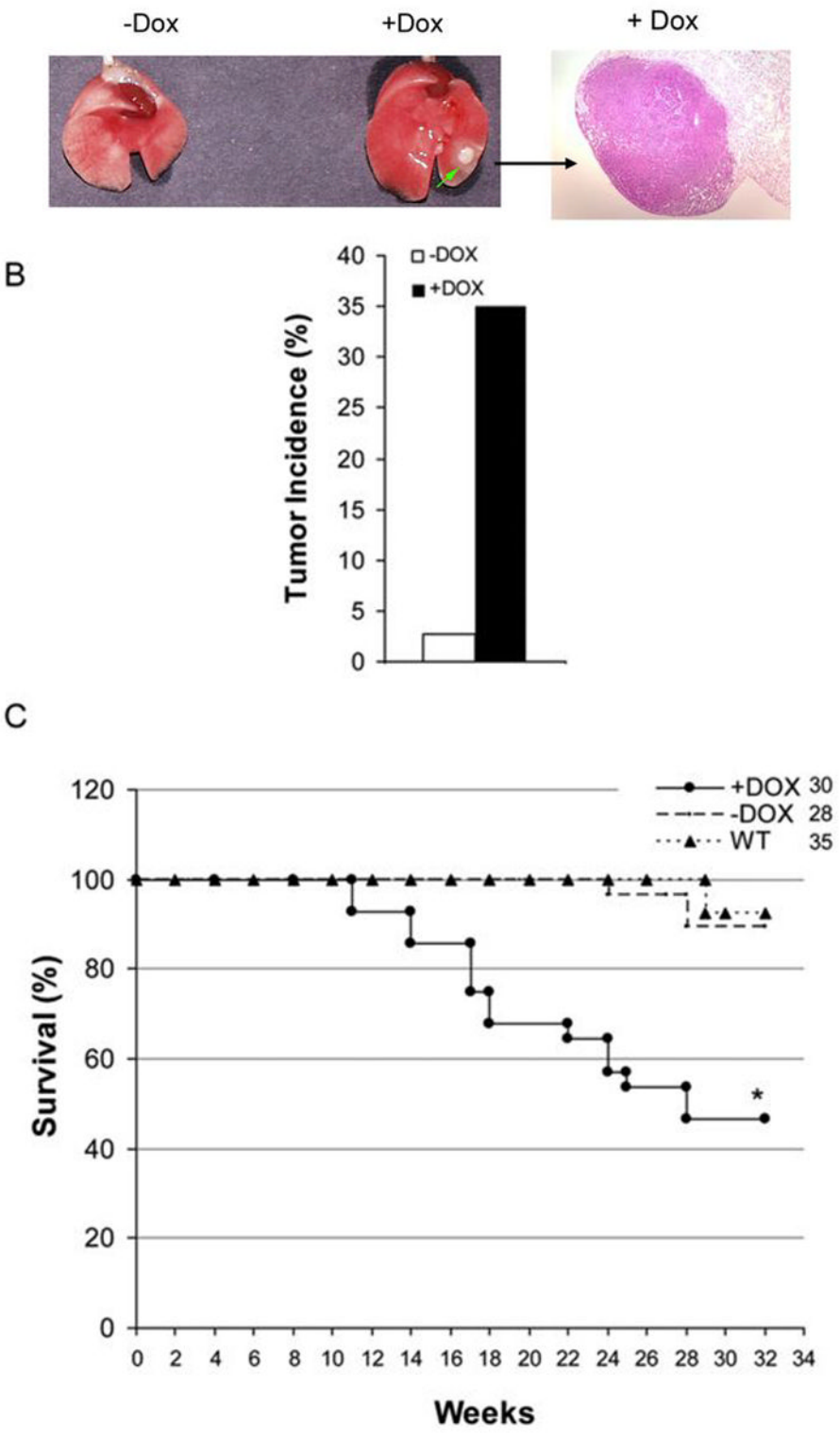
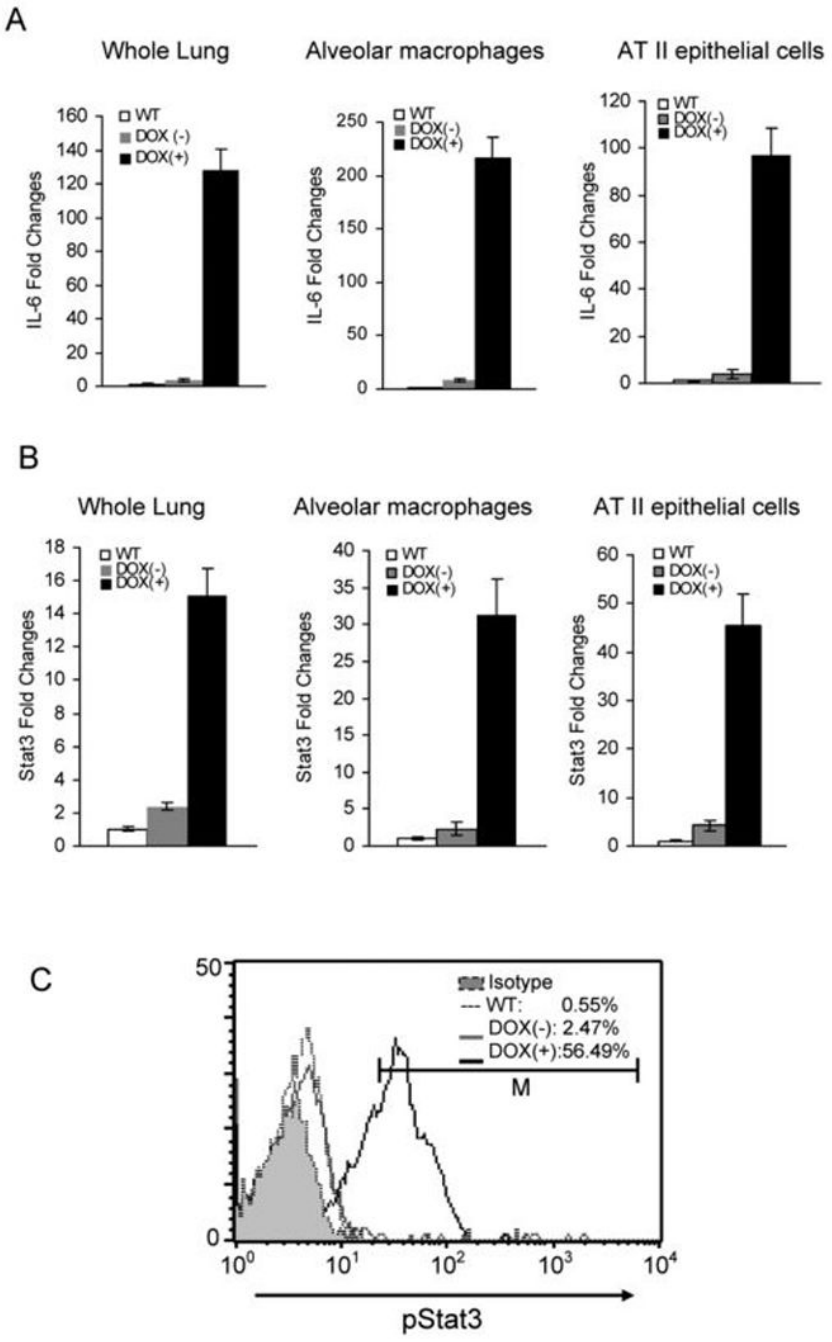


Figure 6.

Overexpression of Api6 caused lung inflammation and bronchoalveolar adenocarcinoma in c-fms-rtTA/(TetO)$_7$-CMV-Api6 bitransgenic mice. **A)** C-fms-rtTA/(TetO)$_7$-CMV-Api6 bitransgenic mice were treated or untreated with doxycycline. Lungs were inflated, sectioned

and analyzed by H&E staining. Bronchioalveolar adenocarcinomas (pointed by the green arrow) were observed in 6-month doxycycline-treated lungs. **B)** Lung carcinoma incidence in 6 to 11-months of doxycycline-treated and untreated bitransgenic mice, n=40. **C)** Animal survival of c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice in 32 weeks of doxycycline-treated or untreated bitransgenic mice. The casualty in each group was recorded. WT, wild type mice (without doxycycline treatment); −DOX, doxycycline-untreated bitransgenic mice; +DOX, doxycycline-treated bitransgenic mice. *, $P< 0.05$.



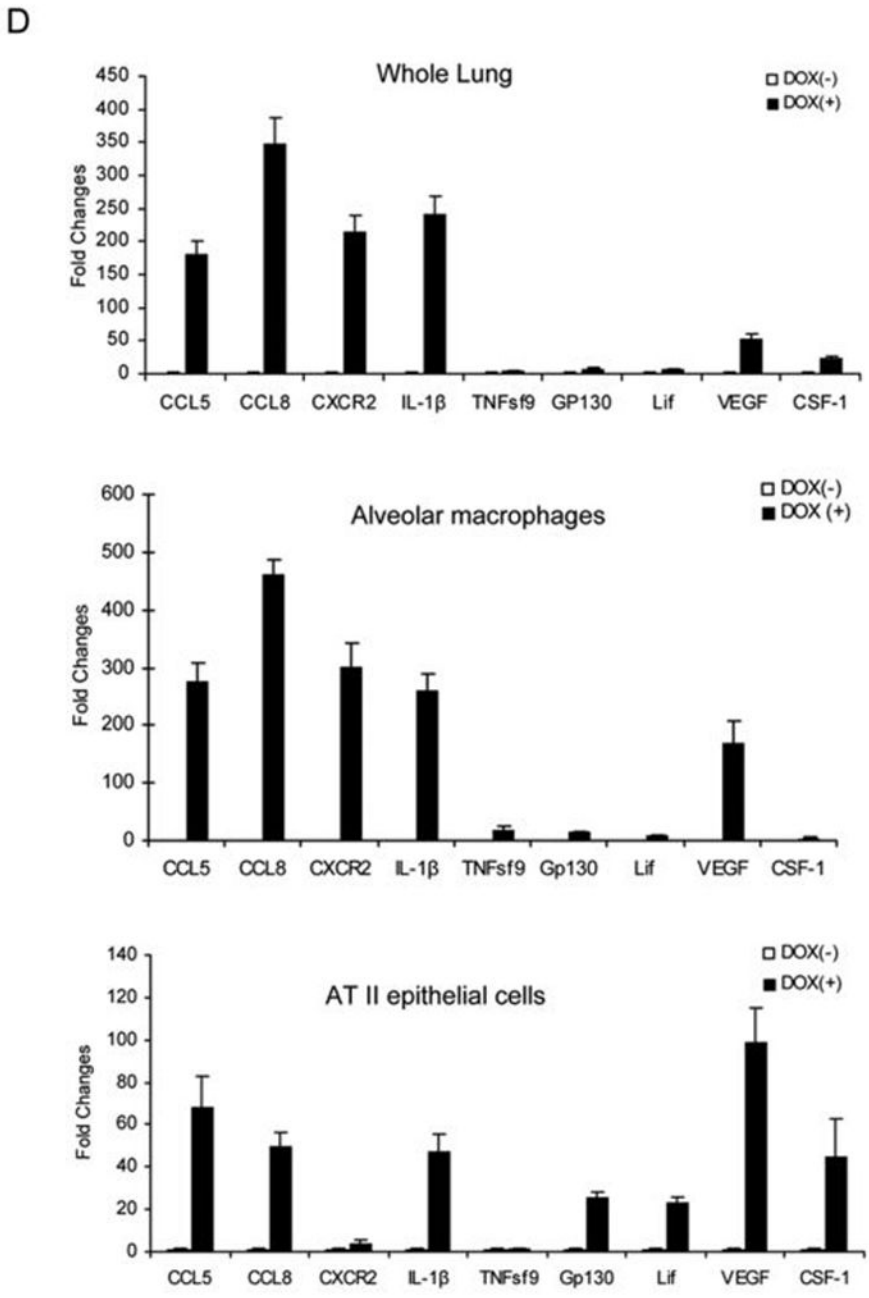


Figure 7.

Overexpression of Api6 increased expression of IL-6 and Stat3 and activation of Stat3 in alveolar type II epithelial cells of c-fms-rtTA/(TetO)$_7$-CMV-Api6 bitransgenic mice. **A-B)** Real-Time PCR analysis of IL-6 and Stat3 in 3-month doxycycline-treated or untreated bitransgenic whole lung, alveolar macrophages and alveolar type II epithelial cells. **C)** The purified lung alveolar type II epithelial cells were stained with SP-C and phosphor-Stat3. Phosphor-Stat3 positive cells were analyzed by flow cytometry in gated SP-C positive cells. **D)** Real-Time PCR analysis of Stat3 downstream cytokines and chemokines in 3-month doxycycline-treated or untreated bitransgenic whole lungs, alveolar macrophages and alveolar type II epithelial cells. In A), B), D), E) and F), data are means of 4 independent experiments ± SD. *P<0.05. WT, wild type mice; DOX (−), doxycycline-untreated bitransgenic mice; DOX

(+), doxycycline-treated bitransgenic mice. The purity of alveolar type II epithelial cells and alveolar macrophages is shown in supplemental Figure 2.

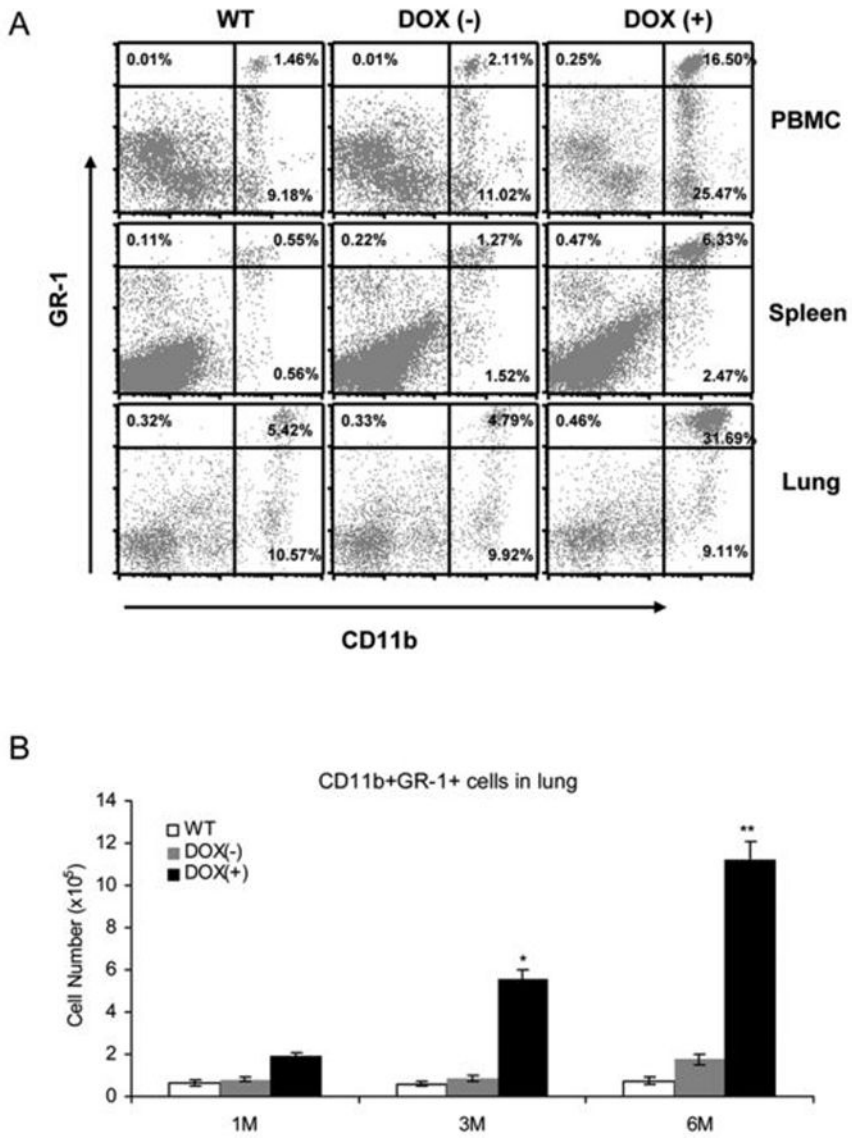
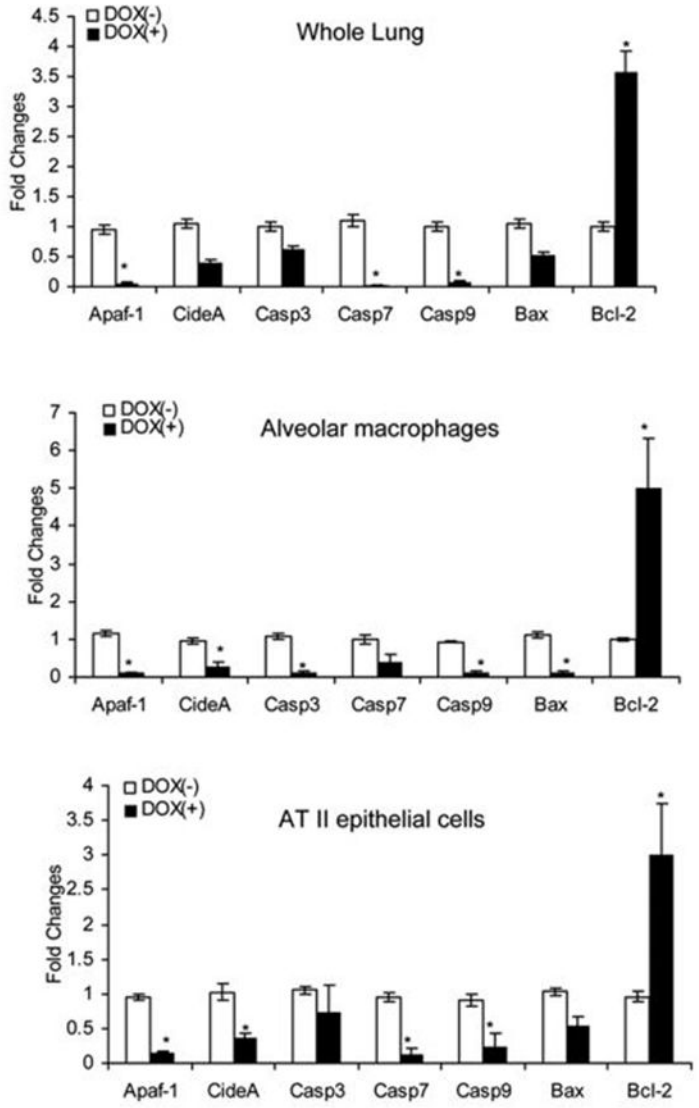


Figure 8.

Overexpression of Api6 increased MDSC population in c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. **A-B)** Single cell suspensions were prepared from the blood (PBMC), spleen and lung of 1, 3, 6-month (1M, 3M, 6M) doxycycline-treated, untreated bitransgenic mice and age-matched wild type mice. The percentage number of Gr-1+CD11b+ cell population was analyzed by FACS in dot plots. Total cellularity was determined by using a hemocytometer and trypan blue exclusion of dead cells. Absolute numbers of cells in each cell subset were determined by multiplying the total number of cells x % live gated x % CD11b, GR-1 or CD11c subsets. A representative dot plot analysis of 3-month doxycycline-treated, untreated bitransgenic mice and age-matched wild type mice is presented (**A**). Isotype control is illustrated in supplemental Figure 6. The time-dependent study of total Gr-1+CD11b+ cells in the lung of doxycycline-treated, untreated bitransgenic mice and age-matched wild type mice is presented in (**B**). Data represent the mean and SD of at least four independent animals (n=4). WT, wild type mice; DOX (−), doxycycline-untreated bitransgenic mice; DOX (+), doxycycline-treated bitransgenic mice.

A



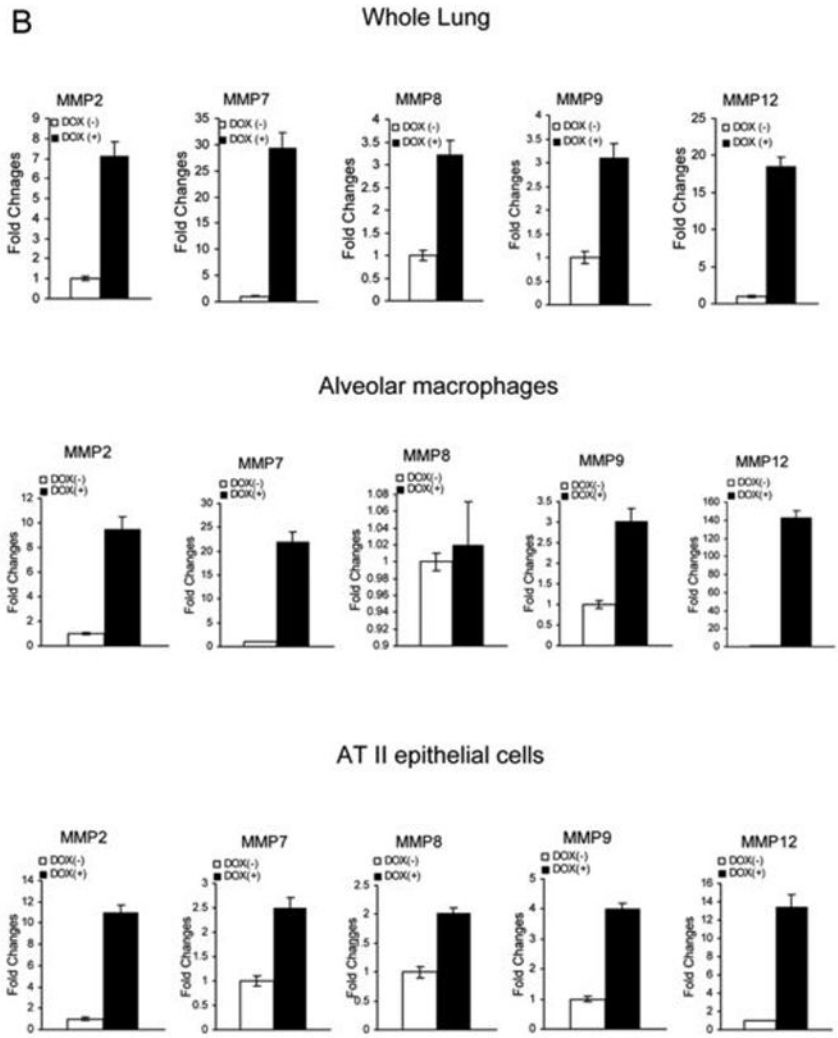


Figure 9.

Overexpression of Api6 altered gene expression of apoptotic molecules, MMPs in the lung of c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice. Total mRNAs were isolated from whole lungs, alveolar macrophages and alveolar type II epithelial cells of 3-month doxycycline-treated or untreated bitransgenic mice. **A)** Real-Time PCR analysis of apoptotic molecules; **B)** Real-Time PCR analysis of MMPs. Each data represents 3 independent (n=3) studies. Dox (−), doxycycline-untreated bitransgenic mice; Dox (+), doxycycline-treated bitransgenic mice.

Table I

Expression of intracellular signaling molecules in myeloid cells from c-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice

		pStat3		pErk		pP38	
		−DOX	**+DOX**	**−DOX**	**+DOX**	**−DOX**	**+DOX**
Bone Marrow	**CD11b**	7.05±2.06	9.44±4.96	3.26±1.72	5.98±2.53	2.78±1.04	6.46±2.04*
	CD11c	2.11±0.58	3.14±1.67	12.29±2.69	16.11±3.20	2.37±0.78	3.96±0.86
	GR-1	7.46±2.01	9.18±1.27	1.48±0.26	3.22±0.93*	1.78±1.09	4.18±1.08*
PBMC	**CD11b**	4.67±1.55	23.32±7.43*	5.77±1.59	40.03±5.62*	5.52±0.92	39.28±9.86*
	CD11c	2.75±0.86	9.43±2.09*	7.71±0.55	11.67±2.17*	10.68±2.37	13.09±3.35
	GR-1	5.87±3.12	16.56±2.80*	6.66±2.73	37.11±5.81*	4.83±1.81	13.27±4.52*
Lung	**CD11b**	2.12±0.82	13.47±4.25*	6.97±2.46	36.14±8.03*	12.46±4.38	40.09±4.83*
	CD11c	1.02±0.49	4.81±2.91*	13.99±2.48	28.34±3.25*	3.26±1.28	8.34±1.94*
	GR-1	1.49±1.36	10.15±1.93*	6.84±2.70	40.75±3.91*	6.51±2.13	19.12±3.10*

DOX(−):doxycycline-untreated bitransgenic mice, DOX(+):doxycycline-treated bitransgenic mice. Percentages of pStat3, pErk or pP38-positive cells in CD11b, CD11c and GR-1 positive cells represent three independent experiments.

*, $P< 0.05$. The specificity of pStat3, pErk and pP38 antibodies is shown in supplemental Figure 7.

Table 2

MFI of intracellular signaling molecules in myeloid cells from C-fms-rtTA/$(TetO)_7$-CMV-Api6 bitransgenic mice

		pStat3		pErk		pP38	
		−DOX	+DOX	−DOX	+DOX	−DOX	+DOX
Bone Marrow	**CD11b**	23.81±3.74	25.4±3.93	19.94±3.08	18.16±1.85	18.64±2.6	28.77±6.53*
	CD11c	20.41±6.93	22.91±10.52	31.57±7.86	31.17±5.25	18.30±3.51	20.04±3.29
	GR-1	25.23±4.07	26.54±1.34	20.09±1.97	24.7±1.1	19.23±4.73	23.76±5.87
PBMC	**CD11b**	23.91±7.69	80.81±23.06*	21.83±7.22	154.59±80.23*	25.12±1.86	158.36±79.83*
	CD11c	21.39±3.73	28.99±7.31	27.76±6.24	28.24±7.34	30.68±10.53	34.85±12.19
	GR-1	23.82±6.46	44.88±12.95*	28.77±7.86	132.92±83.79*	22.01±1.65	32.67±12.16*
Lung	**CD11b**	21.29±4.95	37.84±9.36*	26.47±3.59	120.09±53.15*	36.21±5.77	142.98±67.28*
	CD11c	18.67±4.05	24.51±6.21	32.21±8.31	36.59±11.04	21.71±3.76	27.26±11.76
	GR-1	22.82±4.42	38.46±14.97*	25.95±5.93	132.67±67.90*	22.32±4.15	43.65±22.57*

Comparison of the mean fluorescence intensity (MFI) of pStat3, pErk and pP38 in CD11b+, CD11c+ and GR-1+ cell populations obtained from the bone marrow, PBMC and Lung. The data are mean value ± SD from three independent experiments.

*, P< 0.05.