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Enhanced Th2 cell differentiation and function in the absence of Nox2

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Short title: Enhanced Th2 function without Nox2

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Abstract

Background: Chronic granulomatous disease (CGD) patients, whom inherit abnormal function of NADPH oxidase 2 (Nox2), suffer from hyper inflammatory responses in lung as well as bacterial and fungal infection. There have been studies to reveal the function of Nox2 in hyper-inflammatory diseases, especially in asthma, but the exact role of Nox2 in asthma is still unclear and controversial. Therefore, we attempted to clarify the exact role of Nox2 in asthma, using various experimental asthma models.

Methods: Asthma phenotypes were analyzed in response to various allergen-induced experimental asthma using Nox2 deficient mice and recombinaase gene activating-1-deficient mice. To understand the underlying mechanisms of exaggerated Th2 effector functions, we investigated the degree of T cell activation, levels of activation induced cell death (AICD), and regulatory T (Treg) cell differentiation in Nox2-deficient T cells.

Results: Asthma phenotypes were increased through enhanced Th2 differentiation and function in Nox2-null mice regardless of dose and route of various allergens. Nox2-deficient T cells also showed hyper-activation, reduced AICD, and diminished Treg cell differentiation through increased AKT phosphorylation (T308/S473) and enhanced mitochondrial ROS production.

Conclusion: Our findings indicate that Nox2 deficiency results in exaggerated experimental asthma, which is caused by enhanced Th2 effector function in a T cell intrinsic manner.

Keywords: AKT, Allergic asthma, Mitochondrial ROS, Nox2, Th2 cell

Introduction

Chronic granulomatous disease (CGD) is a rare genetic disorder caused by defects in function of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) (1). Nox2 is a member of Nox family of proteins comprised of Nox complexes 1 through 5, and DUOX 1 and 2 (2). These enzymes share the ability to transport electrons and produce reactive oxygen species (ROS), but their expression patterns and accessory components differ (2). The Nox2 (GP91phox) complex consists of membrane-associated flavin cytochrome b558, including the gp91phox and p22phox subunits, and three cytosolic factors, p47phox, p67phox, and the regulatory GTPase, Rac (3). Expressed mainly in phagocytes including macrophages and neutrophils, the Nox2 complex is often referred to as phagocyte NADPH oxidase (4). The ROS generated by Nox2 exhibit potent anti-pathogenic actions mediating destruction of engulfed microorganisms (4, 5). Therefore, CGD patients are prone to be severely susceptible to life-threatening infections by bacteria and fungi. In addition, they also suffer from hyper-inflammatory responses, which are found in many organs such as skin, liver, gastrointestinal tract and lung (6). Nevertheless, although information on innate immunity regarding infections has been abundantly obtained, many features of pathophysiology on the hyper-inflammatory disease, especially asthma, remain unclear and controversial.

Asthma is a complicated immune-related disease with many clinical features including airway hyper-responsiveness (AHR), hyper-mucus production, and inflammatory cell infiltration (7). The causes of asthma are environmental factors such as allergen disclosure and infection in genetically predisposed individuals (8). Recently, it has become clear that much of the pathology in asthma is related to allergic disorders reflecting repeated allergen-exposure and chronic inflammation (9). Many features of allergic asthma are thought to show the importance of Th2 cell-dominant immune responses to allergens (10). In the pathogenesis of asthma, allergen-sensing Th2 cells migrate to the lung where they secrete IL-4 and IL-13 consequently induce goblet cell hyperplasia and hyper-responsiveness in smooth muscle and epithelial cells of the airway epithelium. In addition, through secretion of IL-5, Th2 cells can recruit eosinophils resulting in airway eosinophilia (9, 10).

Although there have been many attempts to demonstrate the role of Nox2 in asthma pathophysiology by using gp91*phox* (Nox2) deficient mice, these reports have critical limitations (11-14). Firstly, they showed opposite results, in which some studies have revealed increased lung inflammation in Nox2 null mice (11-13), but other study has demonstrated reduced lung inflammation without Nox2 (14). Secondly, those reports had a shortage for confirmation of phenotypes and pathophysiology of asthma in CGD mice, because (i) they exploited just one asthma model which is OVA/Alum and OVA challenge protocol, nevertheless, they showed conflicting results (11, 14), and (ii) they didn't utilize a typical asthma model that type 2 immunity including eosinophilia and Th2 activation play important roles in asthma pathogenesis. In detail, *Banerjee et al* infected *Aspergillus fumigatus* to examine the response of lung inflammation in CGD mice (12) and *Schappi et al* used cell wall of *A. fumigatus* to check up the hyper-inflammatory responses in lung (13). These studies reported that neutrophil and alveolar macrophage mediated inflammatory responses were elevated in Nox2 null mice, not dependent on Th cells. Finally, those studies had a shortage of clear mechanism especially on the Th cell differentiation and activation (11-14). Another previous study showed that in the Nox2 deficiency, increased Th17 differentiation occurs and results in aging-dependent spontaneous arthritis, as well as increased Th2 and reduced regulatory T (Treg) cell differentiation

(15). However, they couldn't define the exact signaling mechanism in a T cell-intrinsic manner and show direct evidence about relation of reduced Treg cell differentiation with disease phenotype.

Protein kinase B (AKT), a serine/threonine kinase, has been known to promote cell proliferation, activation, survival and growth in Th cell (16). In particular, recent studies have verified that (i) activation of AKT mediates Th2 cell differentiation and (ii) inhibition of AKT phosphorylation ameliorates allergic airway inflammation (17, 18). Moreover, several reports have identified AKT as a negative regulator on Treg cell differentiation (19, 20). Recently, association between AKT and mitochondrial respiratory capacity regarding cell apoptosis and oxidative energy synthesis has been studied (21, 22). In these study, they showed that hyper-activated AKT induces up-regulation of mitochondrial respiratory capacity, resulting in resistance to apoptosis and increases glycolysis in cancer cells. In addition to classical role of ROS produced from mitochondria as ATP synthesizer and cellular responder to hypoxia, new insight that mitochondrial ROS in Th cell is critical not only for T cell receptor (TcR)-mediated signaling, but also for IL-2 and IL-4 production has been suggested (23, 24). However, little is known about the relation of Nox2, AKT and mitochondrial ROS.

In this study, we aimed to dissect the exact role of Nox2 in asthma pathogenesis by using various allergic asthma models to clarify controversial arguments. We also attempted to define the exact mechanism in a T cell intrinsic aspect, using in vitro T cell activation with Nox2 deficient mice. We confirmed enhanced allergic lung inflammation in Nox2-deficient mice compared to wild type (WT) mice. Furthermore, we showed that Nox2-deficient Th cells exerted hyper-activation, diminished activation induced cell death (AICD) and reduced Treg cell differentiation through highly activated AKT-increased mitochondrial ROS production axis in a T cell-intrinsic manner. Therefore, our results clarify the controversial results of previous studies and demonstrate the exact mechanism for hyper-inflammatory response especially in lung of CGD patients, using CGD mice. Further, our study suggests regulation of Nox2 could be a strategy to control hyper-inflammatory disease beyond allergic asthma.

Materials and methods

Mice

Detailed procedure is described in Data S1.

Allergens and other reagents

Allergens and reagents were purchased from various vendors. Detailed information regarding this is provided in Data S1.

In vitro T cell stimulation

In vitro T cell stimulation was performed as described previously (25). Detailed procedure is described in Data S1.

Allergen administration and analysis of asthma phenotypes

Asthma induction and analysis were performed as previously described (26, 27). For all saline and allergen-challenged mice, BAL cytology, glycoprotein, and lung histopathology were determined as previously described (28). Detailed information is present in Data S1.

Measurement of secreted glycoprotein in BAL fluid

Secreted glycoprotein in BAL fluid was quantified, as previously described (27). Detailed procedure is described in Data S1.

Adoptive transfer of CD4 T cells

Detailed procedure is described in Data S1.

Quantitation of cytokine-producing cells

Detailed procedure is described in Data S1.

Flow Cytometry

Flow cytometry was performed as described before and a detailed protocol is described in Data S1 (28).

In vitro and in vivo Treg differentiation

In vitro and in vivo Treg differentiation was performed as described previously (29, 30). A detailed procedure was described in Data S1.

Protein extraction and western blot analysis

The detailed protocol is described in Data S1.

Treg functional assay

The detailed protocol is described in Data S1.

Statistics

The detailed protocol is described in Data S1.

Results

Exaggerated allergic lung inflammation in Nox2-deficient mice

Because there have been debates on the effects of Nox2 deficiency in allergic asthma pathogenesis (11-14), we sought to investigate the degree of allergic lung inflammation in Nox2-deficient mice, using various allergens. First, we examined a mixture of *Aspergillus* protease (AP) and chicken egg ovalbumin (OVA) (APO) allergen-induced allergic lung inflammation (26). WT mice with intranasal APO challenge developed full allergic lung inflammation that included AHR, predominant bronchoalveolar lavage (BAL) eosinophilia, increased BAL glycoprotein and IL-4 secretion, and marked parenchymal eosinophilia and airway goblet cell metaplasia (Fig. 1A-F). Compared to WT mice, Nox2-deficient mice showed exaggerated allergic asthma with significantly enhanced AHR, airway inflammation, BAL mucus, IL-4 and IL-13 hyper-production and increased airway goblet cell metaplasia (Fig. 1A-F). Furthermore, to confirm these enhanced allergic inflammation was due to increased Th2 differentiation and function in Nox2-deficient mice, we examined IL-4 and GATA3 expression by Th cells upon OVA re-stimulation. As expected, Nox2-null mice showed enhanced IL-4 and GATA3 expression compared to WT mice (Fig. 1G-I). In addition, largely similar results were obtained in the same mice challenged with systemic sensitization of OVA precipitated in aluminum sulfate (OVA/Alum) delivered intraperitoneally and intranasal OVA allergen (Fig. S1).

More importantly, without AP, direct intranasal OVA challenges, which have been known as a tolerance induction model, induced allergic asthma phenotypes only in Nox2-deficient mice (Fig. 2A-F). In contrast, OVA-only challenge in WT mice did not induce phenotypes of allergic lung inflammation (Fig. 2A-F). Remarkably, compared to WT mice that showed OVA-specific tolerance

by induction of lung Treg cells, there was no increased Treg cell differentiation in Nox2-deficient mice (Fig. 2G, H). Lastly, without aluminum sulfate addition, direct intraperitoneal and intranasal OVA administration induced allergic lung inflammation in Nox2 null mice (Fig. S2). Thus, regardless of respiratory allergens and challenge routes, Nox2 deficiency causes development of significant allergic lung inflammation.

Enhanced Th2 immunity of Nox2-null mice is T cell intrinsic

Although Th2 cells play a pivotal role in induction of allergic asthma and our data suggest enhanced immune functions are due to a primary T cell effect, we also considered abnormalities in antigen presenting cells (APCs) and/or phagocyte function that preclude activation of Th cells (31-33). Thus, we conducted additional in vivo studies to rule out the effect of Nox2 deficiency in cells other than T cells. For this purpose, we examined allergic asthma phenotypes in allergen-challenged Rag-1-deficient mice that had received highly purified WT or Nox2-deficient naïve CD4 T cells (Fig. 3A-F). No allergic lung inflammation was observed in allergen-challenged Rag-1-deficient mice without transfer of CD4 T cells (Fig. 3A-E). However, Nox2-deficient T cell transferred Rag-1-null mice showed exaggerated features of allergic parameters (Fig. 3A-F). In contrast, WT CD4 T cells induced moderate asthma phenotypes in Rag-1 reconstituted mice (Fig. 3A-F). Moreover, Rag-1-null mice that received Nox2-deficient CD4 T cells showed numerous CD4 T cells in lung compared to WT CD4 T cell-transferred mice (Fig. 3G, H). Thus, Nox2-deficient CD4 T cells were highly proliferated and activated in Rag-1-null mice. In addition, similar results that CD45.1 WT mice received Nox2-deficient naïve OT-II CD4 T cells showed increased phenotype of asthma compared to mice received WT OT-II CD4 T cells were obtained (Fig. S3). Enhanced allergic lung inflammation was thus induced in a manner dependent on provision of Nox2-deficient CD4 T cells, and independent of Nox2 and/or ROS expression by non-T cells. Taken together, these results indicate that the CD4 T cell-specific defect of Nox2 is responsible for the elevated allergic inflammation.

Enhanced in vitro Th cell function without Nox2

As shown in previous results, Nox2 deficiency caused increased allergic lung inflammation via enhanced Th2 function. To confirm the effect of Nox2 deficiency on Th2 differentiation in vitro, we performed in vitro experiments under various conditions. First, relative to WT T cells, naïve Nox2-deficient CD4 T cells activated by cross-linking of pre-coated anti-CD3 and soluble anti-CD28 antibodies proliferated more and secreted higher amounts of IL-2 and IL-4 during primary stimulation (Fig. 4A-C). These data indicate that naïve CD4 T cells have a tendency to hyper-activate without Nox2 nor ROS in T cell-intrinsic manner. Next, to avoid the developmental and indirect effects of Nox2 deficiency, we firstly activated WT CD4 T cells with pre-coated anti-CD3 and soluble anti-CD28 antibodies in the presence of gp91ds-tat, which has been known as a specific Nox2 inhibitor unlike apocynin and diphenylene iodonium (DPI) to knock-down Nox2 (34, 35). Consistent with previous data, WT CD4 T cells treated with gp91ds-tat proliferated more and produced higher amounts of IL-4 (Fig 4D, E). Secondly, we added irradiated T cell-depleted WT splenocytes as APCs during T cell stimulation with soluble anti-CD3 and CD28 antibodies and found similar results (Fig. S4). We also examined in vitro T cell activation without Nox2 in an antigen-dependent manner. To do so, we crossed Nox2-deficient mice with OT-II mice to generate OVA peptide-specific CD4 T cells. Similar to antibody stimulation, Nox2-deficient OT-II cells showed enhanced proliferation and cytokine production (Fig. S5). Lastly, we activated Nox2-deficient and WT naïve CD4 T cells with a minimal stimulation by adding anti-CD3 antibody without APCs. Again, hyper-proliferation and increased IL-4 production were detected only in Nox2-deficient T cells (Fig. 4F, G). Furthermore, decreasing amounts of anti-CD3 antibody stimulation clearly demonstrate that Nox2 deficiency consistently enhanced naïve CD4 T cell activation. Thus, these data suggest that upon primary stimulation, enhanced proliferation and cytokine secretion are observed in Nox2-deficient Th cells.

T cell activation is prolonged in the absence of Nox2

To demonstrate the precise signaling mechanism of enhanced Th2 differentiation without Nox2, we examined several adaptor molecules involved in TcR-mediated signaling. There was increased phosphorylation of AKT (T308 and S473), which plays a critical role in mediating Th2 differentiation in Nox2-deficient T cells, confirming hyper-activation (Fig. 5A) and consistent with previous study (17, 18). However, phosphorylation of ZAP70 and LCK was not different (data not shown). Moreover, we hypothesized that mitochondrial ROS, which can function as a signaling enhancer (23, 24) might be up-regulated with Nox2 deficiency, because there have been reports that (i) AKT activation enhances mitochondrial ROS production and (ii) Nox2 deficiency might be compensated through increased mitochondrial ROS production to fulfill the increased energy demand by glycolysis (21, 22). To test this possibility, we assessed the level of mitochondrial ROS in WT and Nox2-deficient T cells. Compared with WT, Nox2-deficient CD4 T cells stimulated with anti-CD3 and CD28 antibodies produced higher amounts of mitochondrial ROS although cellular ROS production was low (Fig. 5B, C and Fig. S6). To confirm increased mitochondrial ROS production is a direct cause of enhanced Th2 differentiation, we pre-treated Nox2-deficient CD4 T cells with MitoQ and/or rotenone, mitochondria-specific antioxidant and electron transport chain inhibitor, and measured IL-4 production and T cell proliferation. As expected, treatment of Nox2-deficient CD4 T cells with MitoQ or rotenone diminished IL-4 production and proliferation to levels similar to that seen in antibody-stimulated WT CD4 T cells (Fig. 5D, E).

Lastly, to evaluate the effects of increased mitochondrial ROS in exacerbated allergic lung inflammation, we administered MitoQ and/or rotenone intra-tracheally half hour before APO challenge. Nox2-deficient mice with treatment showed similar levels of asthma phenotypes to that seen in WT mice (Fig. 6A-D). To confirm the similar asthma phenotypes through reduced Th2 differentiation, total numbers of IL-4 and/or IL-13 producing cells and levels of IL-4 and IL-13 (thus defined as Th2 cells) were determined by ELISpot and ELISA assays. Levels of IL-4 and IL-13 were diminished by treatment of MitoQ or rotenone compared to vehicle challenged mice (Fig. 6E-I). In addition, populations of CD4⁺CD25⁺ cells in lung (thus defined as activated T cells) were decreased

by treatment with MitoQ or rotenone (Fig. 6J). Although intratracheal administration of MitoQ or rotenone could lead to non-specific effects to other cells such as eosinophils, we could conclude that CD4 T cells require mitochondrial ROS for induction of increased lung inflammation in Nox2 null mice rather than other cells, especially eosinophils do because previous studies demonstrate that mitochondrial ROS induce apoptosis of eosinophils (36, 37), but treatment of MitoQ or rotenone resulted in decreased numbers of eosinophils in BAL fluid in contrast with the previous results. Thus, anti-mitochondrial ROS effects with MitoQ or rotenone treatment seems to cause decreased eosinophilia through negative effects on CD4 T cell activation and Th2 cell differentiation rather than non-specific effects. In summary, increased mitochondrial ROS with Nox2 deficiency would be a direct reason for increased allergic lung inflammation via enhanced Th2 differentiation.

Reduced AICD and Treg cell differentiation in Nox2-null mice

Because the levels of cellular ROS, which induce AICD through induction of FasL and down-regulation of Bcl-2 were relatively low (38), and activation of AKT, which induces resistance to apoptosis was up-regulated without Nox2 (16), we assumed that reduced cell death could be another molecular mechanism of prolonged CD4 T cell activation without Nox2. However, we also consider the effects of increased mitochondrial ROS in Nox2 deficient condition, which potentially promote cell death (39). For this, we conducted experiments to examine the conflicting effects of Nox2 deficiency and increased mitochondrial ROS on AICD of CD4 T cells. Firstly, we examined the levels of AICD depend on time phases and confirmed that AICD were significantly reduced in Nox2 deficiency (Fig. 7A, B). Interestingly, Nox2 deficient CD4 T cells exerted reduced AICD in late phase but not in early time point. Secondly, to evaluate the precise effects of mitochondrial and total cellular ROS on AICD, we measured the levels of AICD after pre-treatment of MitoQ and rotenone which are mitochondrial specific or N-acetyl-cysteine (NAC), a cellular intracellular free-radical scavenger. The levels of AICD were significantly reduced in WT CD4 T cells with NAC and mitochondrial specific reagents treatment and similar to that seen in treated Nox2 deficient CD4 T

cells in early phase (Figure S7), but no significant differences were observed by either MitoQ or rotenone treatment in late phase (Fig 7C, D). Based on our present results, we could conclude that mitochondrial ROS could be a crucial trigger but not sufficient to promote cell death in overall phases, which requires sustained accumulation of intracellular ROS by the action of Nox2. In other words, decreased levels of cellular ROS with Nox2 deficiency is a direct cause of reduced AICD rather than enhanced mitochondrial ROS in overall phases. Our results are consistent with previous studies that mitochondrial ROS inhibition by rotenone treatment results in not only block of sequential Nox activation but also reduced cell death only in early phase not overall phases (40-42). In addition, these results could explain the similar levels of AICD in early phase between WT and Nox2 deficient CD4 T cells, which means that increased pro-apoptotic effects through enhanced mitochondrial ROS might compensate the AICD in spite of decreased levels of cellular ROS without Nox2. In conclusion, diminished AICD through reduced cellular ROS production could be another mechanism underlying the prolonged Th2 cell function in Nox2-null mice. In addition to these molecular mechanisms, another cellular mechanism is a reduced Treg cell function through higher AKT phosphorylation, which suppresses Treg cell differentiation in these mice (19, 20). Indeed, previous study demonstrates decreased Treg cells could be a mechanism of spontaneous aging dependent arthritis in Nox2-deficient mice (15). To confirm decreased Treg differentiation directly mediates increased allergic asthma phenotypes and Th2 differentiation, we assessed in vitro and in vivo Treg cell differentiation. Compared to WT T cells, Nox2-deficient CD4 T cells gave rise to decreased numbers of Treg cells, simultaneously with enhanced IL-4 production (Fig. S8). Moreover, pre-treatment of MitoQ or rotenone resulted in increased Treg differentiation compared to vehicle group (Fig. S8). Furthermore, it is consistent with previous studies that (i) Treg cells engage in fatty acid oxidation metabolic pathway during their differentiation, whereas Th2 cell differentiation is engaged in glycolysis, and (ii) ROS generated from mitochondria are essential for glycolysis in T cell activation (43). Consistent with in vitro results, in vivo Treg cell differentiation was also diminished in lungs of intranasal OVA-challenged Nox2-deficient mice, which have been known to induce tolerance (30, 44), along with induction of asthma phenotypes (Fig. S8). However, both WT- and Nox2-deficient Treg cells exhibited normal function as conventional Treg functional assay showed

similar results (Fig. S9). Therefore, the selective failure of Treg cell function provides an explanation for the increased inflammatory status of Nox2-deficient mice.

Discussion

In this study, we clarify the effects of Nox2 deficiency in allergic asthma pathogenesis, using CGD mice. Our study demonstrates that Nox2-deficient mice lead to exacerbated allergic lung inflammation regardless of allergen or challenge route. In addition, we also verify that Nox2 deficiency in CD4 T cells results in their hyper- and prolonged-activation due to increased phosphorylation of AKT (T308/S473) and mitochondrial ROS production as well as decreased AICD and Treg differentiation. Furthermore, increased mitochondrial ROS production without Nox2 could be a direct cause of enhanced allergic asthma through increased Th2 differentiation. Therefore, our results indicate mitochondrial ROS as well as Nox2 could be targets for the development of therapeutics against Th2-dependent diseases including asthma.

Nox2 was first described in phagocytes and originally considered to have a very restricted role in bactericidal activity (5). However, in parallel with advances in understanding of phagocyte Nox2, it was found that Nox2 is also expressed in non-phagocytic cells including hepatocytes, endothelial cells, hematopoietic cells, neurons, B cells, and T cells (2). Thus, recent studies have attempted to reveal the role of Nox2 in T cells. These studies suggested that Nox2 deficiency elevates Th1 and Th17 differentiation resulting in not only improved pathogen containment but also aging-dependent spontaneous arthritis (15, 45). Furthermore, diverse reports tried to define the effects of Nox2 deficiency in allergic disease, but their conclusions are controversial (11-14). Based on these results, we attempted to clarify the role of Nox2 in asthma pathogenesis and its precise mechanism especially in regulation of Th2 cell differentiation. Interestingly, our study leads to the clear finding that asthma phenotypes and Th2 activation are elevated in Nox2-null mice, using various asthma models. Further, we revealed that Nox2-deficient CD4 T cells are hyper-activated due to increased phosphorylation of AKT on T308 and S473 residues, an event important for Th2 differentiation and

Treg suppression (17-20). We also discovered that in the early phase of TcR stimulation, mitochondrial ROS was increased in Nox2-deficient cells. Thus, increased mitochondrial ROS would be an explanation for the hyper-activation in these mice. This conclusion is consistent with previous findings that mitochondrial ROS in CD4 T cell is critical for not only TcR-mediated signaling but also IL-2 and IL-4 production (23, 24). In addition to hyper-activation of Th2 cells, we also detected diminished Treg differentiation and AICD without Nox2. Thus, these results imply that Nox2-deficient naïve CD4 T cells intrinsically tend to be Th2 effector cells.

Based on our findings, we conclude that exacerbated asthma is caused by hyper- and prolonged-activation of Th2 cells, resulting from highly phosphorylation of AKT and increased production of mitochondrial ROS as well as decreased Treg differentiation and AICD. Thus, we carefully analyzed the correlation between increased mitochondrial ROS and Nox2 deficiency. Regarding this, we reasoned that increased mitochondrial ROS is caused by AKT activation and compensation for the absence or reduction of cellular ROS. In other words, (i) enhanced phosphorylation of AKT would induce mitochondrial respiratory capacity for glycolysis and (ii) increased mitochondrial ROS satisfies extra energy demands to compensate defects of cellular ROS, which plays a role in glycolysis during T cell activation, resulting in an increased metabolic status. This is consistent with previous reports of enhanced activation without Nox2 due to elevated mitochondrial function (43, 46). Nonetheless, further studies need to clarify the precise molecular function of Nox2 in mitochondrial ROS and cytokine production of CD4 T cells.

Previous studies that used Nox2 inhibitors, apocynin and DPI and a ROS scavenger, NAC suggest beneficial effects of Nox2 inhibition or ROS scavenging for the alleviation of allergic lung inflammation (31, 32). However, these approaches have a significant limitation because Nox2 inhibition or ROS scavenging is not T cell specific and overfull dose of treatment could result in nonspecific effects and cell toxicity (34). Moreover, these studies are not consistent with previous reports that CGD patients suffer from hyper-inflammatory disease and bacterial and fungal infections (6). On contrary, our study takes advantage of Nox2-deficient mice and adoptive transfer models with Rag-1-deficient mice to clarify the precise role of Nox2 in T cell-specific manner. Furthermore, we

utilized gp91ds-tat to knock-down Nox2 to confirm the role of Nox2 in CD4 T cell activation without developmental and secondary effects, and verified that WT CD4 T cells treated with the specific Nox2 inhibitor show similar levels of proliferation and IL-4 production to those seen in Nox2-deficient CD4 T cells. We also took advantage of MitoQ or rotenone to clarify the role of mitochondrial ROS in Th2 differentiation and asthma pathogenesis without affecting cytosolic ROS. Pretreatment with MitoQ or rotenone not only reduced IL-4 production and cell proliferation but also restored Treg differentiation in Nox2-deficient T cells to levels similar to that seen in WT T cells. Furthermore, we investigated the elevated pro-apoptotic potential of mitochondrial ROS on AICD without Nox2, using MitoQ or rotenone. Although, in early time phase after activation, increased mitochondrial ROS could offset the reduced cellular ROS through Nox2 deficiency, it is not enough to induce cell death in overall phase. In addition, treatment of MitoQ or rotenone decreased asthma phenotypes through diminished Th2 differentiation in Nox2-null mice to levels similar to that in WT mice. Based on these results, we conclude increased mitochondrial ROS by Nox2 deficiency would be a direct molecular mechanism of enhanced allergic lung inflammation and Th2 function. Our data are also consistent with a recent paper using Nox2-deficient mice showing that Th2 and Th17 differentiation are augmented in association with spontaneous and aging-dependent arthritis (15).

Considerable effort has been made to identify asthma treatment for both quick relief of symptoms and long-term suppression of airway inflammation. In addition, other immunotherapeutic approaches such as down-regulation of immunoglobulin E and DNA vaccination have been tried (47). Nevertheless, whether used alone or in combination, these therapies have various side effects and limited efficacy in some patients. Based on our study, Nox2 is a crucial mediator of Th2 regulation and, by extension, allergic asthma symptoms. Furthermore, specific regulation of Nox2 in CD4 T cells represents a novel immunotherapeutic strategy for allergic diseases including allergic asthma, allergic rhinitis and atopic dermatitis.

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Author contributions

Conception and design: BIK, EKJ, CHL, SHL; Analysis and interpretation: BIK, TWK, KS, YK, CMY, JMY, DMS, SHL; Drafting the manuscript for important intellectual content: BIK, SHL.

Conflicts of interest

The authors declared no conflict of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplemental materials and methods.

Figure S1. Exaggerated asthma symptoms in Nox2 deficient mice.

Figure S2. Enhanced asthma phenotypes in Nox2 null mice.

Figure S3. Augmented asthma phenotypes in Nox2 null CD4 T cell reconstituted mice.

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Figure S4. Hyper-activation of naïve CD4 T cells without Nox2.

Figure S5. Enhanced activation of OT-II T cells in the absence of Nox2.

Figure S6. Decreased cytosolic ROS production in Nox2 deficiency.

Figure S7. Diminished AICD by mitochondria specific anti-oxidants treatments in early phase.

Figure S8. Reduced Treg differentiation in the absence of Nox2.

Figure S9. Normal Treg cell function in the absence of Nox2.

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Figure legends

Figure. 1. Enhanced asthma phenotypes in Nox2-deficient mice, as shown by APO challenge. AHR was measured with the flexiVent system (A, ○: WT saline, □: Nox2^{-/-} saline, ●: WT APO, ■: Nox2^{-/-} APO), and eosinophilia (B), glycoprotein secretion (C), IL-4 and IL-13 production (D, E) in BAL fluid were examined. Sections of the lungs were stained with PAS (F). Arrowheads indicate metaplastic goblet cells; arrows indicate inflammatory cells (Original magnification was 400×). IL-4⁺ CD4 T cells were assessed by flow cytometry (G). GATA3 expression was assessed by western blot analysis and densitometry (H, I). Data are presented as mean ± SEM, ** $p < 0.005$ and * $p < 0.05$ compared with the WT APO-challenged group and are representative of three independent experiments (n = 3, □: saline, ■: APO).

Figure. 2. Increased asthma phenotypes in Nox2 null mice challenged with OVA alone. AHR (A, ○: WT saline, □: Nox2^{-/-} saline, ●: WT OVA, ■: Nox2^{-/-} OVA), BAL eosinophilia (B), BAL glycoprotein (C), BAL IL-4 and IL-13 levels (D, E) were assessed (□: saline, ■: OVA). Lung sections were stained with PAS to evaluate goblet cells (F). Arrowheads indicate metaplastic goblet

cells; arrows indicate inflammatory cells (Original magnification was 400×). (G, H) Treg cells in lung were assessed by flow cytometry. Representative (G) and compiling data (H) are shown (□: saline, ■: OVA). Data are representative of three independent experiments (n = 4) and are presented as mean ± SEM, *** $p < 0.0005$, ** $p < 0.005$ and * $p < 0.05$ compared with WT OVA challenged group.

Figure 3. Elevated allergic inflammation in Rag-1 null mice reconstituted with Nox2-deficient CD4 T cells. AHR (A, ○: No transfer saline, ●: No transfer APO, ▲: WT CD4 T cell transfer APO, ▼: Nox2^{-/-} CD4 T cell transfer APO), and eosinophil numbers (B), glycoprotein (C) and the level of IL-4 (D) and IL-13 (E) in BAL fluid were assessed (□: saline, ■: APO). Lung sections were stained with PAS (F). (G, H) Adoptively-transferred populations of CD4 T cells were detected in lungs by flow cytometry. Representative (G) and compiling data (H) are shown (□: saline, ■: APO). Data are presented as mean ± SEM, * $p < 0.05$ compared with Rag-1 deficient mice reconstituted with WT CD4 T cells and are representative of three independent experiments (n = 4).

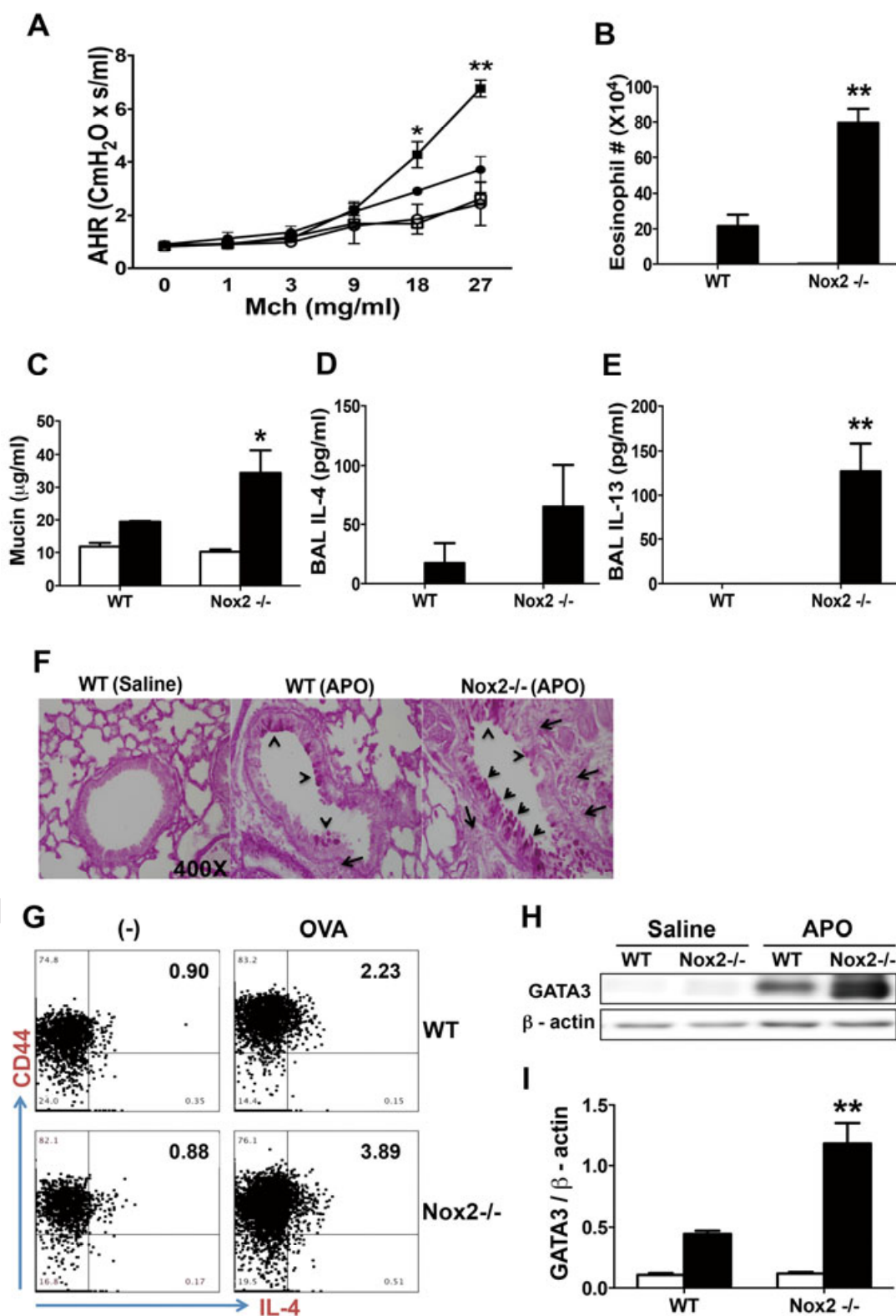
Figure 4. Hyper-activation of Nox2-deficient naïve CD4 T cells with antibody stimulation. Cell proliferation (A), IL-2 (B) and IL-4 (C) production were measured by MTT and ELISA analyses (□ : media, ■: plate bound anti-CD3 and soluble anti-CD28 antibodies). Cell proliferation (D) and IL-4 secretion (E) measured by BrdU incorporation and ELISA after activation with antibody in the presence of gp91ds-tat (□: media, ■: plate bound anti-CD3 and soluble anti-CD28 antibodies). Cell proliferation (F) and IL-4 (G) production assessed by BrdU incorporation and ELISA after serially diluted plate bound anti-CD3 antibody only stimulation (○: WT, ●: Nox2^{-/-}). Data are presented as mean ± SEM, *** $p < 0.0005$, ** $p < 0.005$, and * $p < 0.05$ compared with WT T cells stimulated with antibodies and representative of three independent experiments (n = 3).

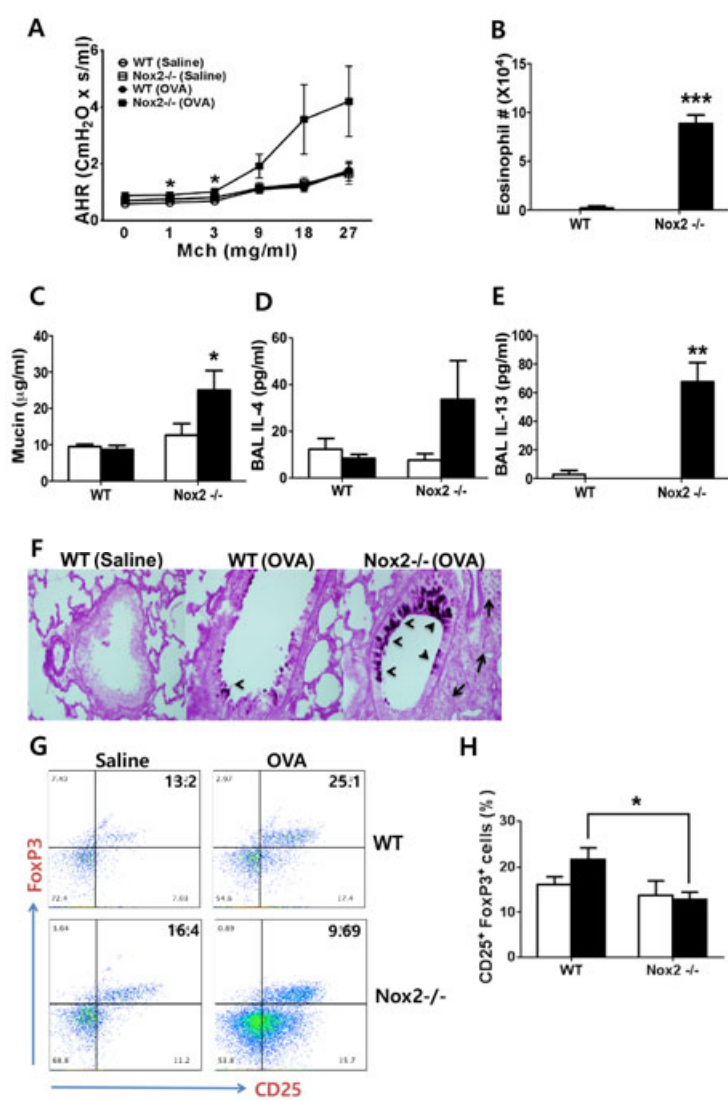
Figure 5. Exaggerated TcR-mediated signaling and elevated mitochondrial ROS in Nox2-deficient T cells. (A) The levels of phosphorylation of Akt (T308 and S473) were assessed by western blot analysis. (B, C) Mitochondrial ROS levels, stained with MitoSOX, were assessed by flow cytometry (□: WT, ■: Nox2^{-/-}). Compiling (B) and representative data (C) are shown. IL-4 production (D) and T cell proliferation (E) were measured by ELISA and BrdU incorporation assays after antibody stimulation and pretreatment with MitoQ or rotenone (□: WT, ■: Nox2^{-/-}). Data are representative of three independent experiments (n=3) and are presented as mean ± SEM, **** $p < 0.0001$, ** $p < 0.005$ and * $p < 0.05$ compared with WT T cells stimulated with antibodies.

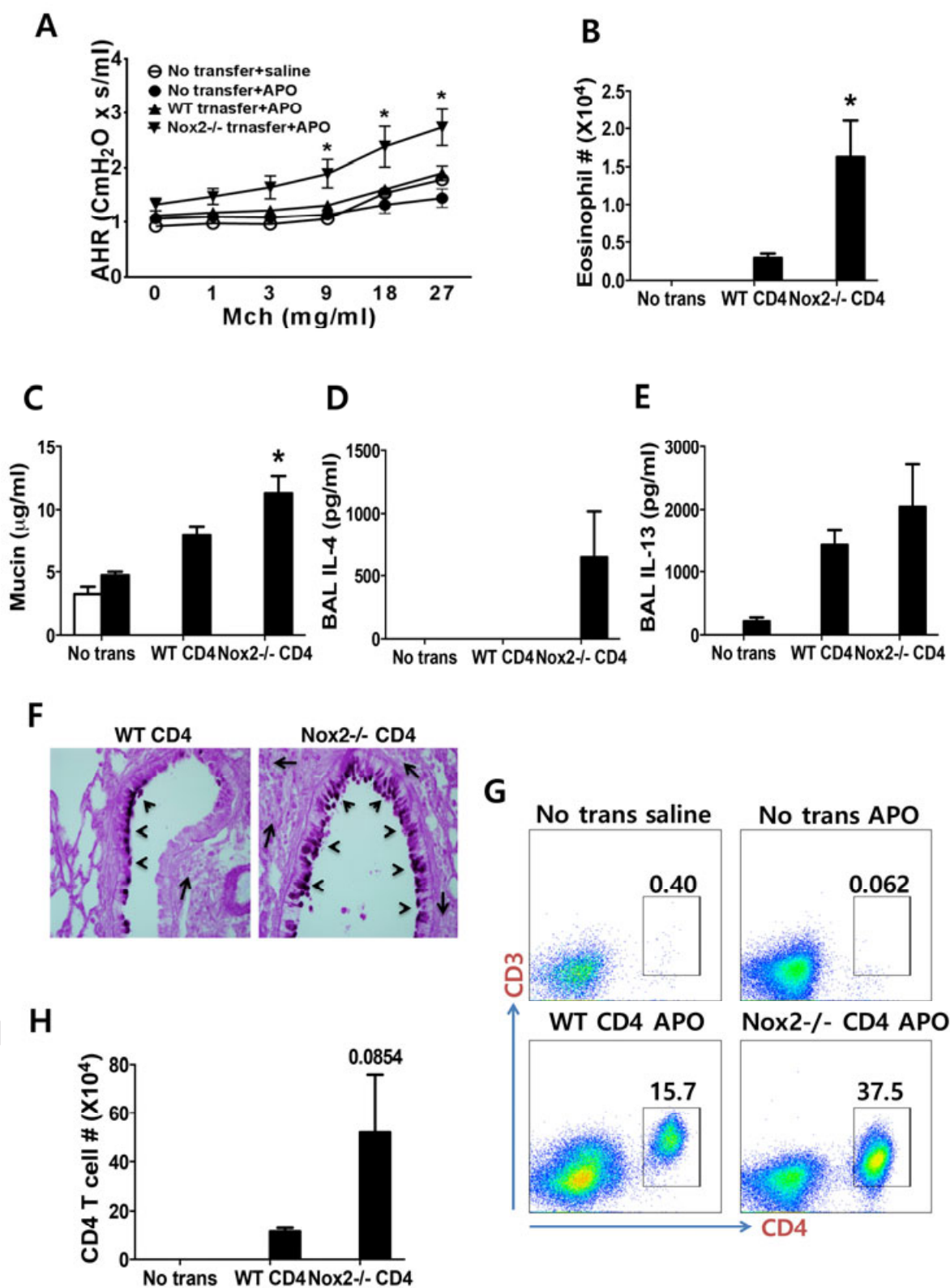
Figure 6. Diminished allergic lung inflammation by treatment of MitoQ and rotenone. Eosinophil numbers (A), glycoprotein (B) and the level of IL-4 (C) and IL-13 (D) in BAL fluid were assessed (□: WT, ■: Nox2^{-/-}). (E-G) Total IL-4 and IL-13 secreting cells in lung were quantified by ELISpot assay. Representative (E) and compiling (F, G) data of IL-4 and IL-13 producing cells are shown (□: WT, ■: Nox2^{-/-}). Levels of IL-4 (H) and IL-13 (I) in lung were assessed by ELISA after soluble anti-CD3 and anti-CD28 antibodies stimulation (□: WT saline, ■: Nox2^{-/-} saline, ▨: WT APO, ▩: Nox2^{-/-} APO, ▪: WT APO + MitoQ, ▫: Nox2^{-/-} APO + MitoQ, ▬: WT APO + rotenone, ▮: Nox2^{-/-} APO + rotenone). Populations of CD4⁺CD25⁺ T cells were detected in lungs by flow cytometry. (J) Data are presented as mean ± SEM, ** $p < 0.005$, * $p < 0.05$ compared with WT challenged mice as indicated and are representative of three independent experiments (n = 3).

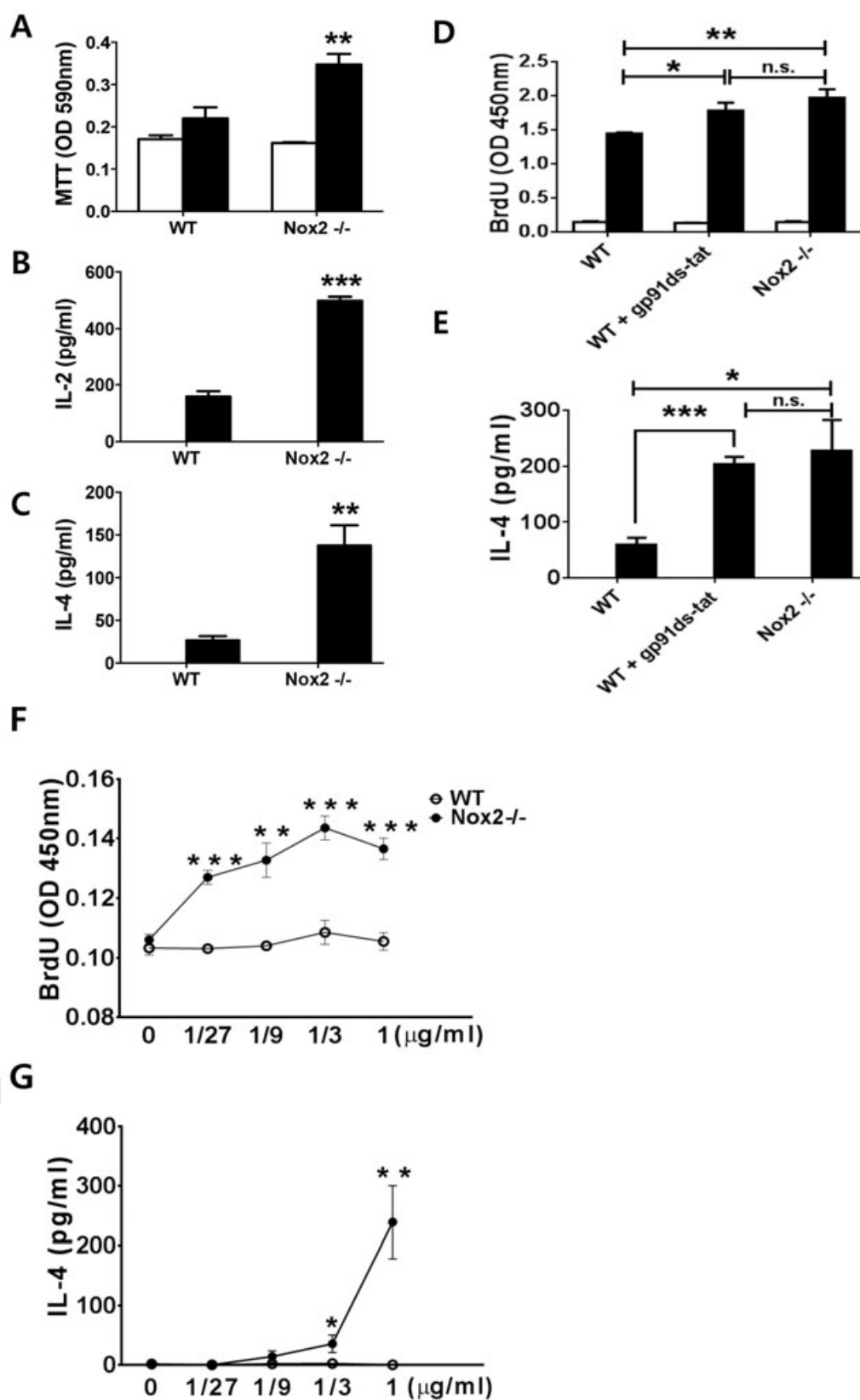
Figure 7. Prolonged T cell activation in the absence of Nox2 through reduced AICD. Caspase-3⁺ CD4 T cells were assessed by flow cytometry. Representative (A) and compiling data (B) are shown (n = 3, □: 2 hours, ▨: 12 hours, ▩: 24 hours, ■: 48 hours). Caspase-3⁺ CD4 T cells were analyzed with or without pretreatment of either NAC, MitoQ or rotenone by flow cytometry after 48 hours with antibody stimulation (n = 3, □: Ab, ▨: Ab + NAC, ▩: Ab + MitoQ, ■: Ab + rotenone). Representative (C) and compiling data (D) are shown. Data are presented as mean ± SEM, **** $p < 0.0001$, ** $p < 0.005$ and * $p < 0.05$ compared with WT challenged mice as indicated and are representative of three independent experiments (n = 3).

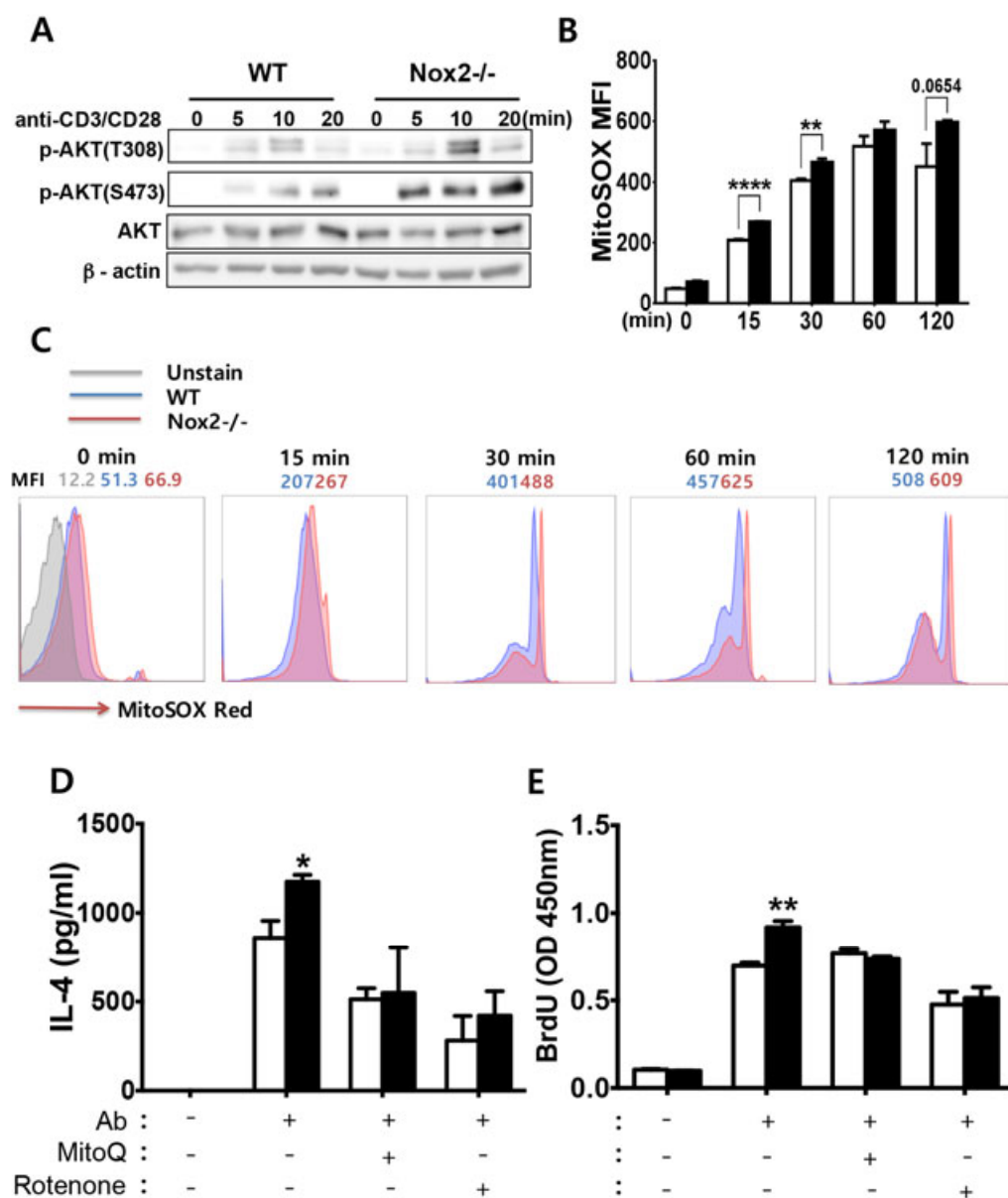
0.005 and * $p < 0.05$ compared with WT antibody stimulation, indicated group and are representative of three independent experiments.

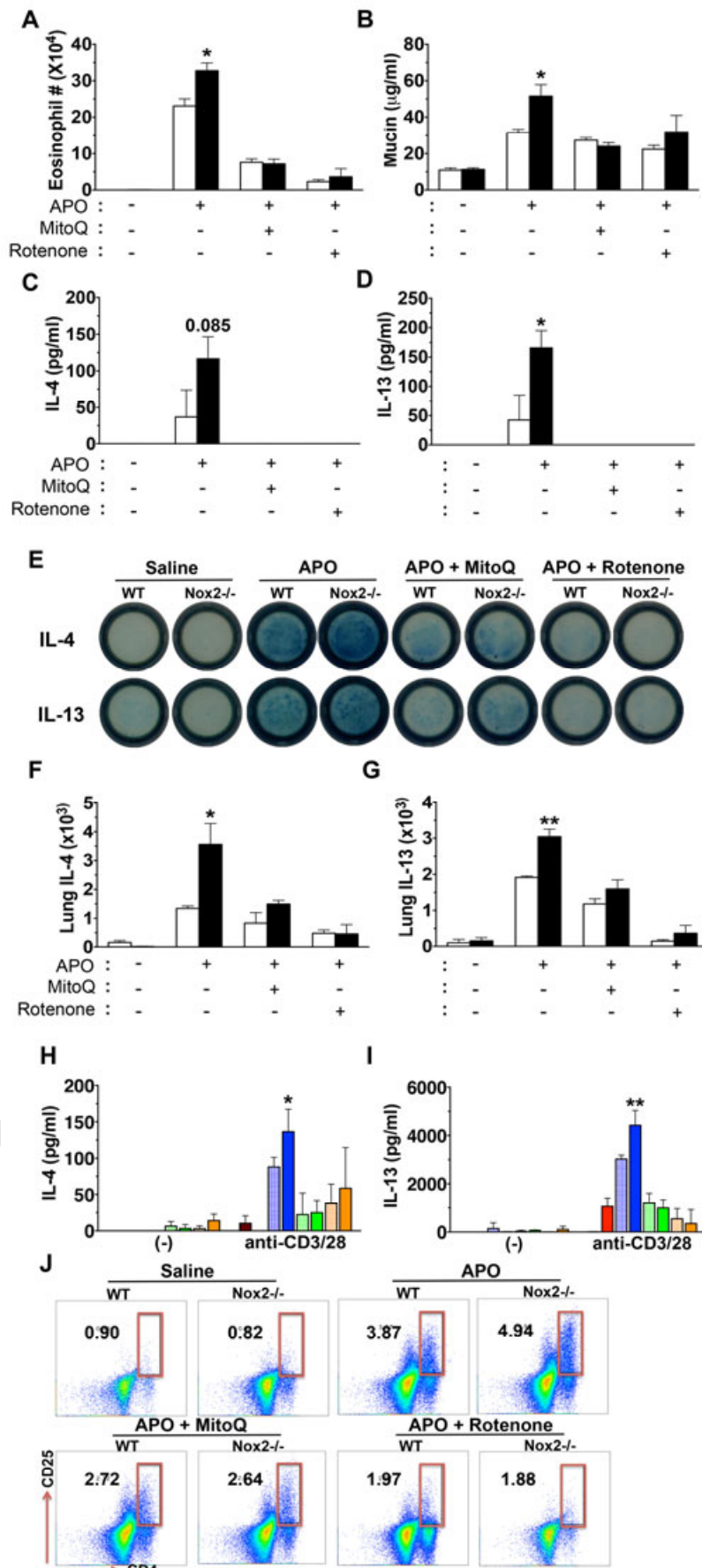


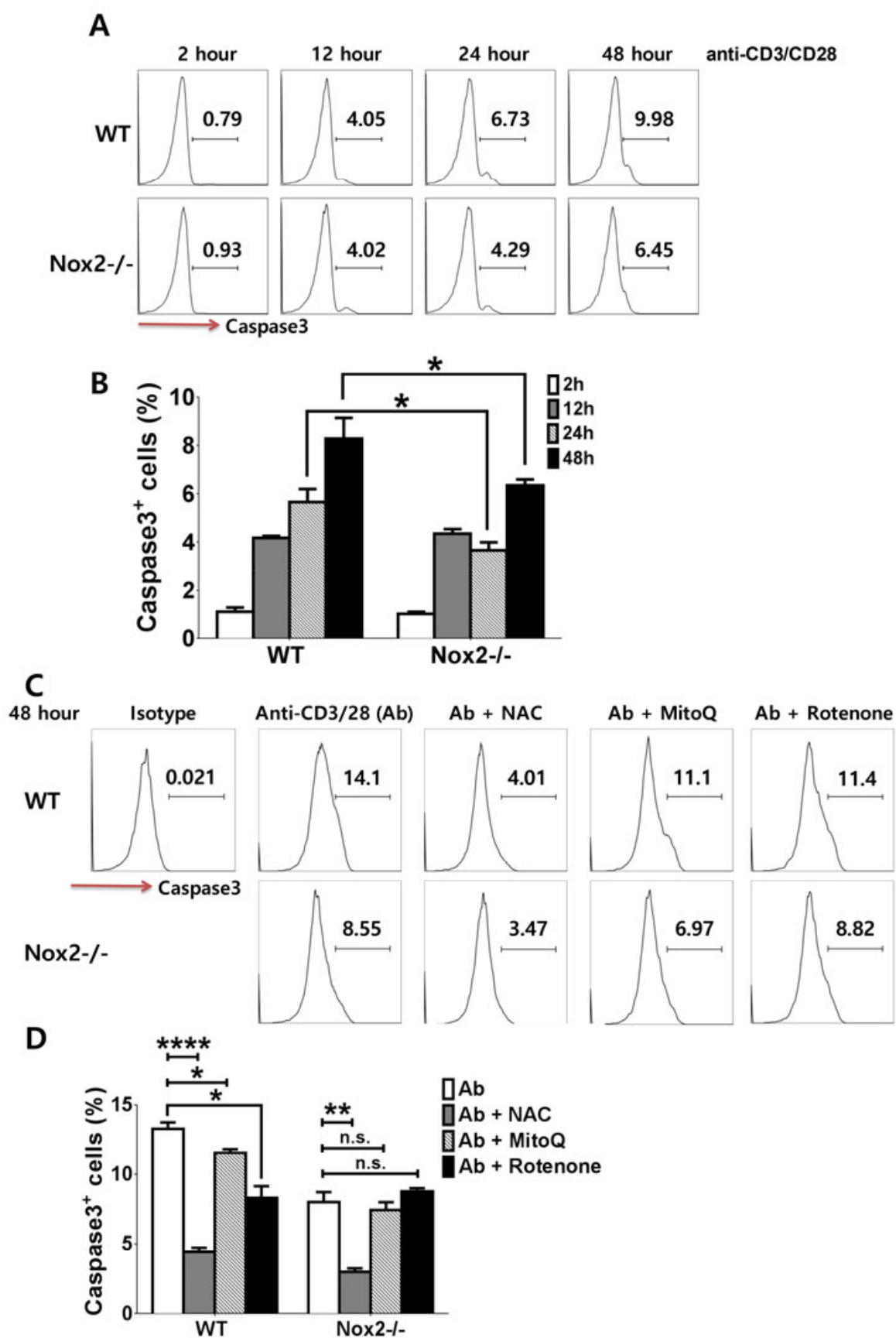












Supplemental Figures

Figure S1

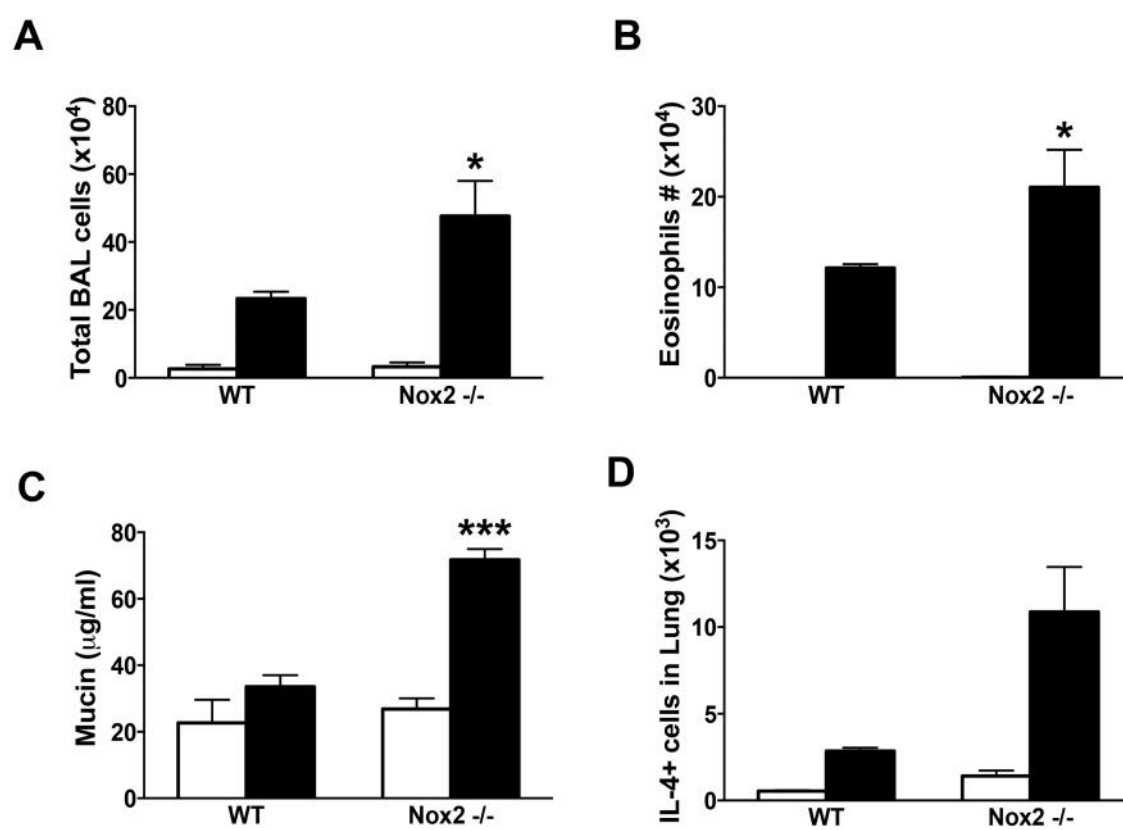


Figure S2

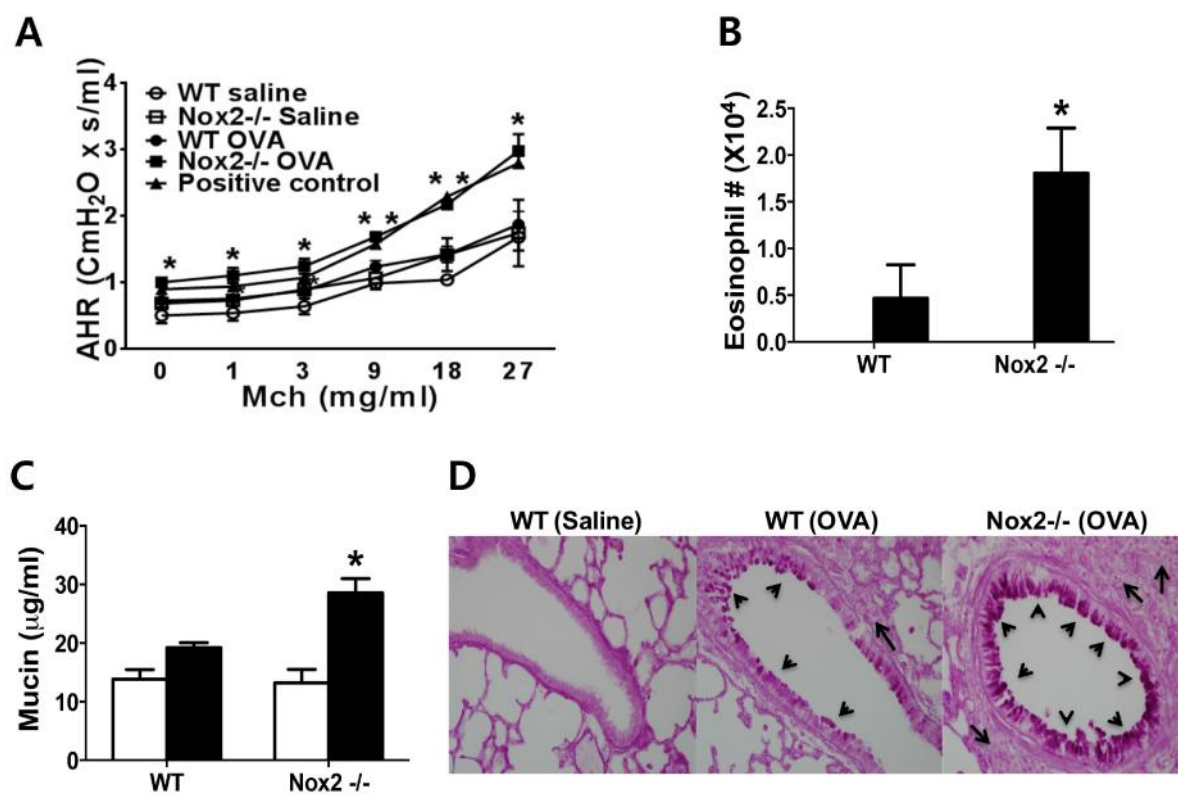


Figure S3

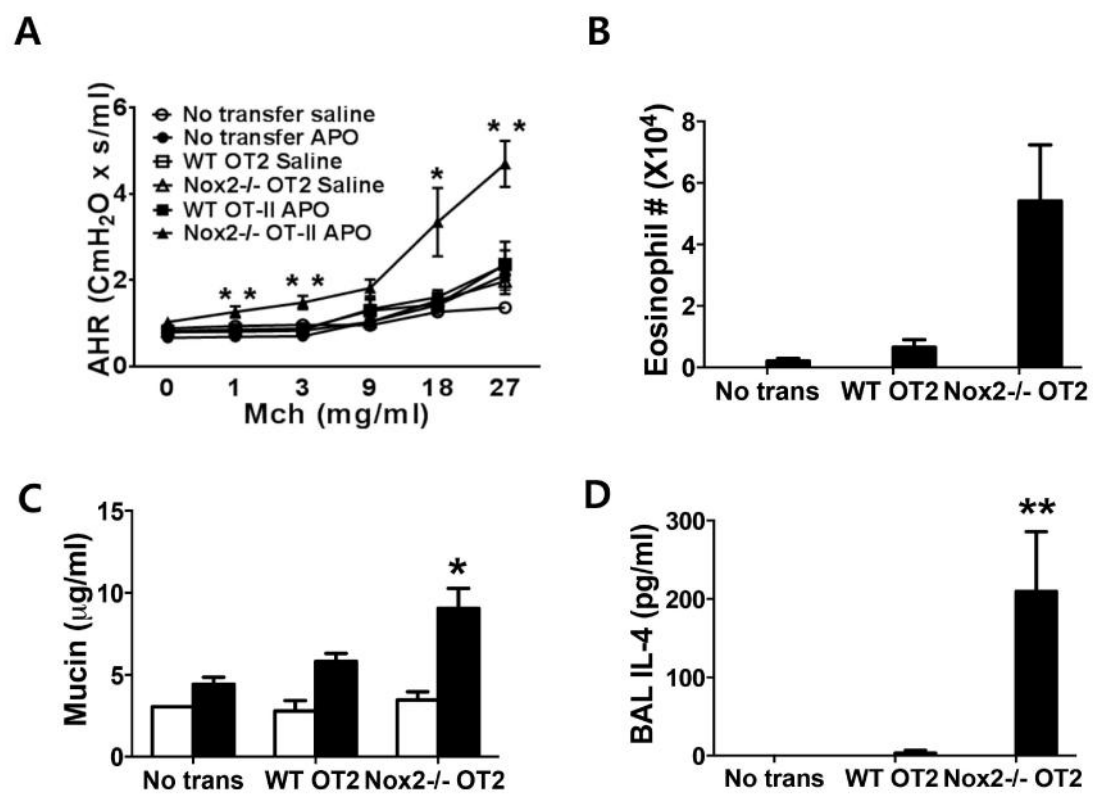


Figure S4

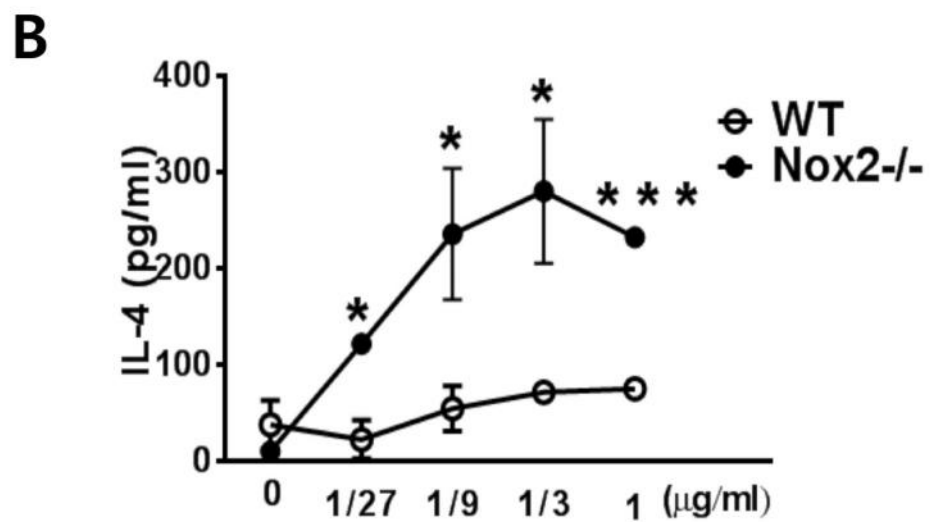
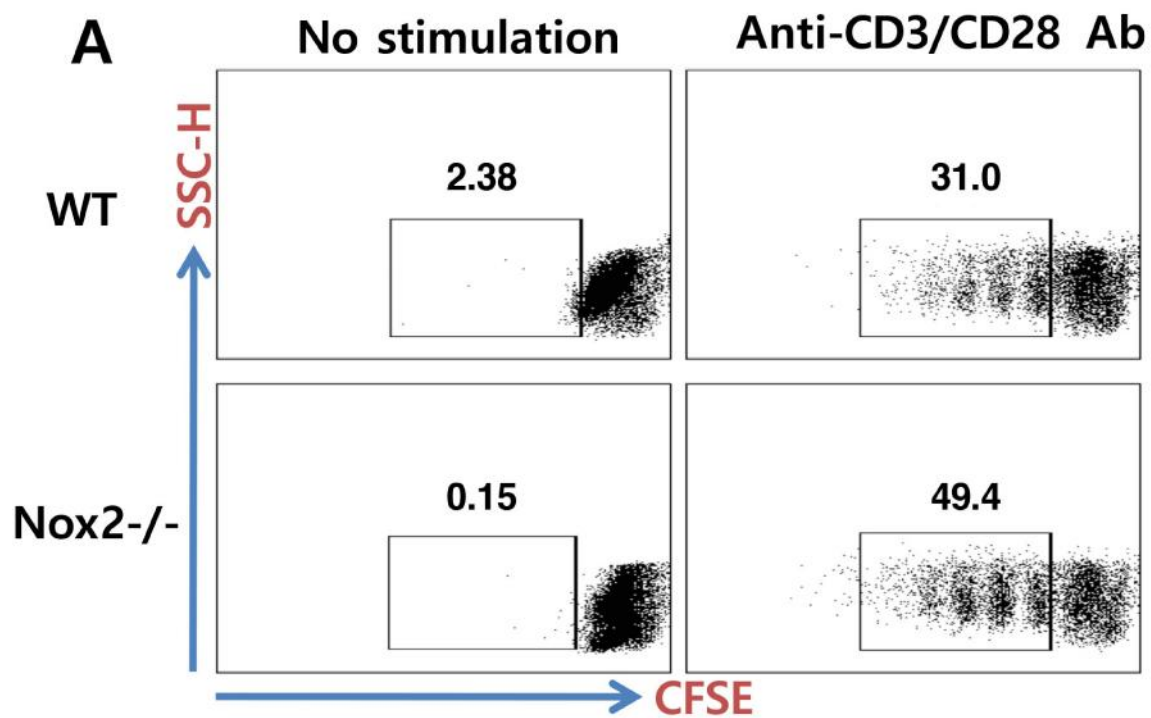


Figure S5

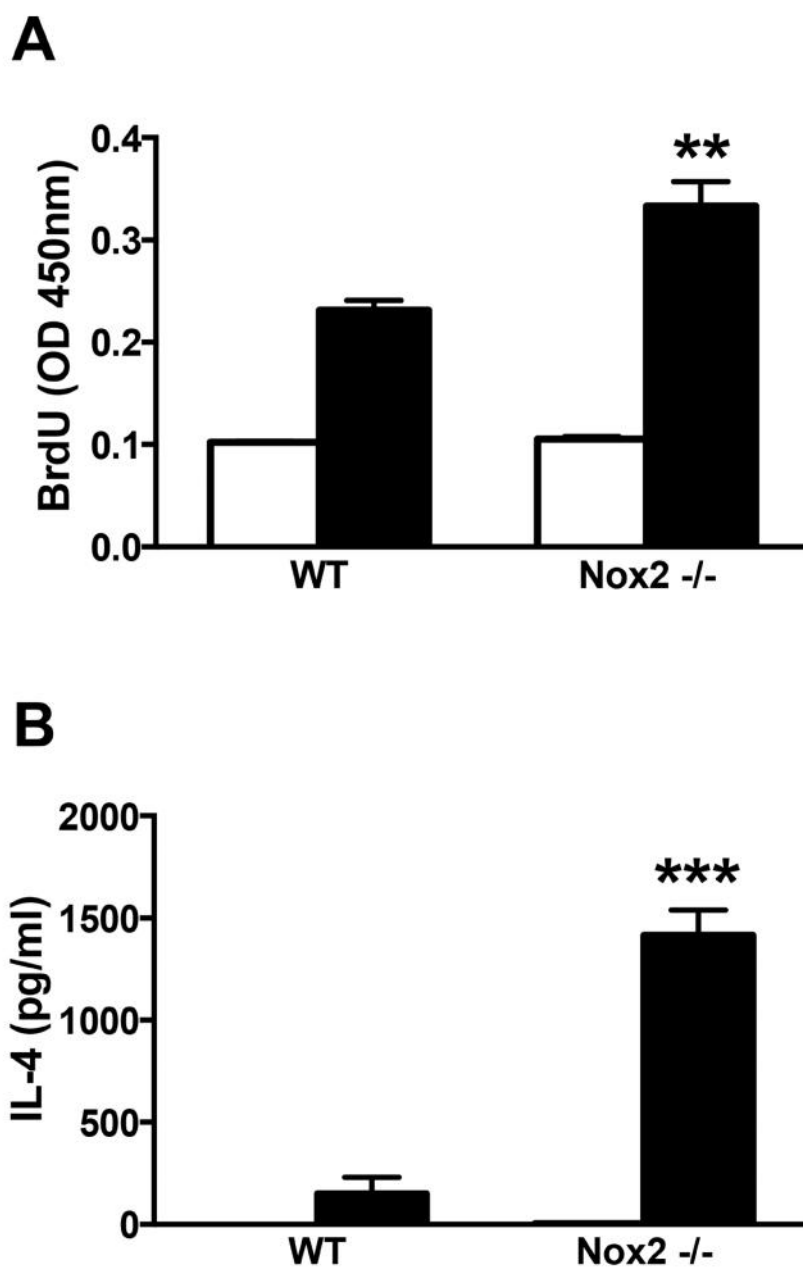


Figure S6

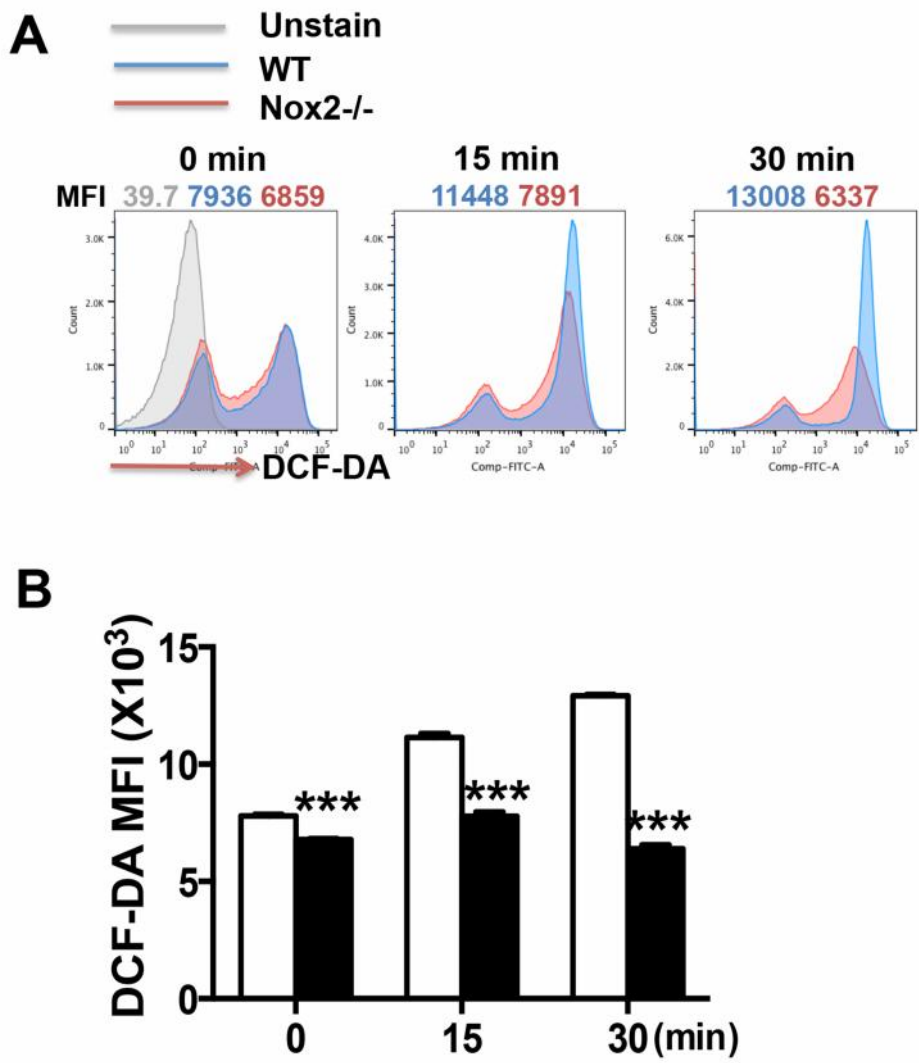


Figure S7

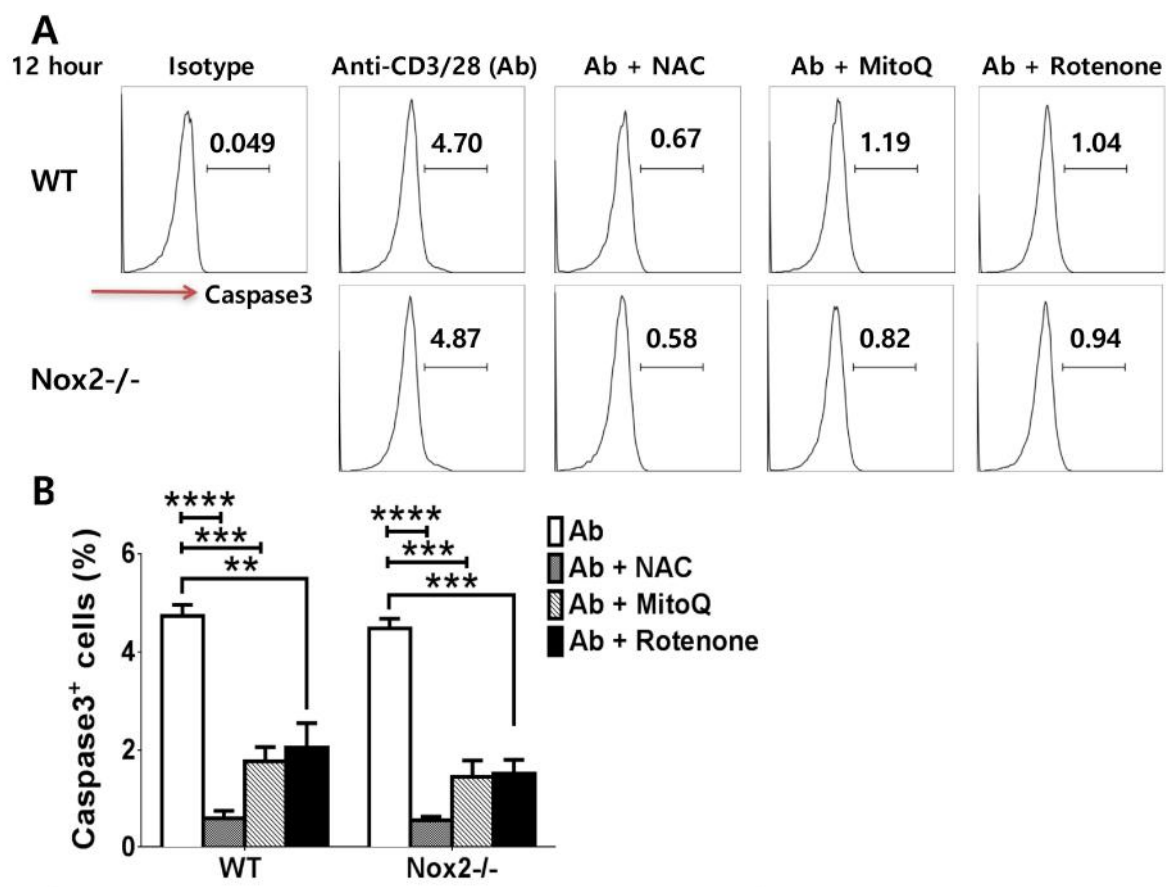
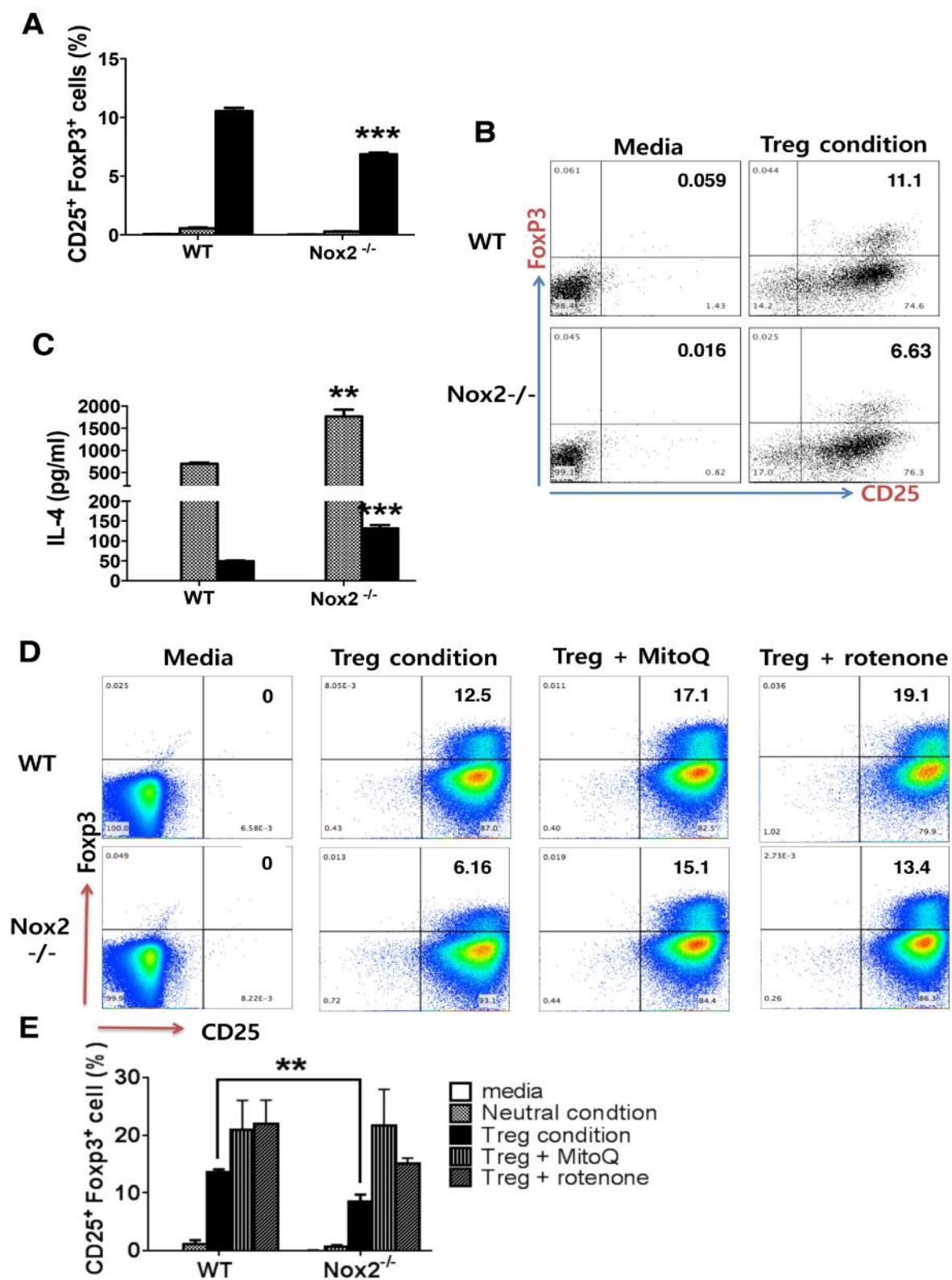


Figure S8



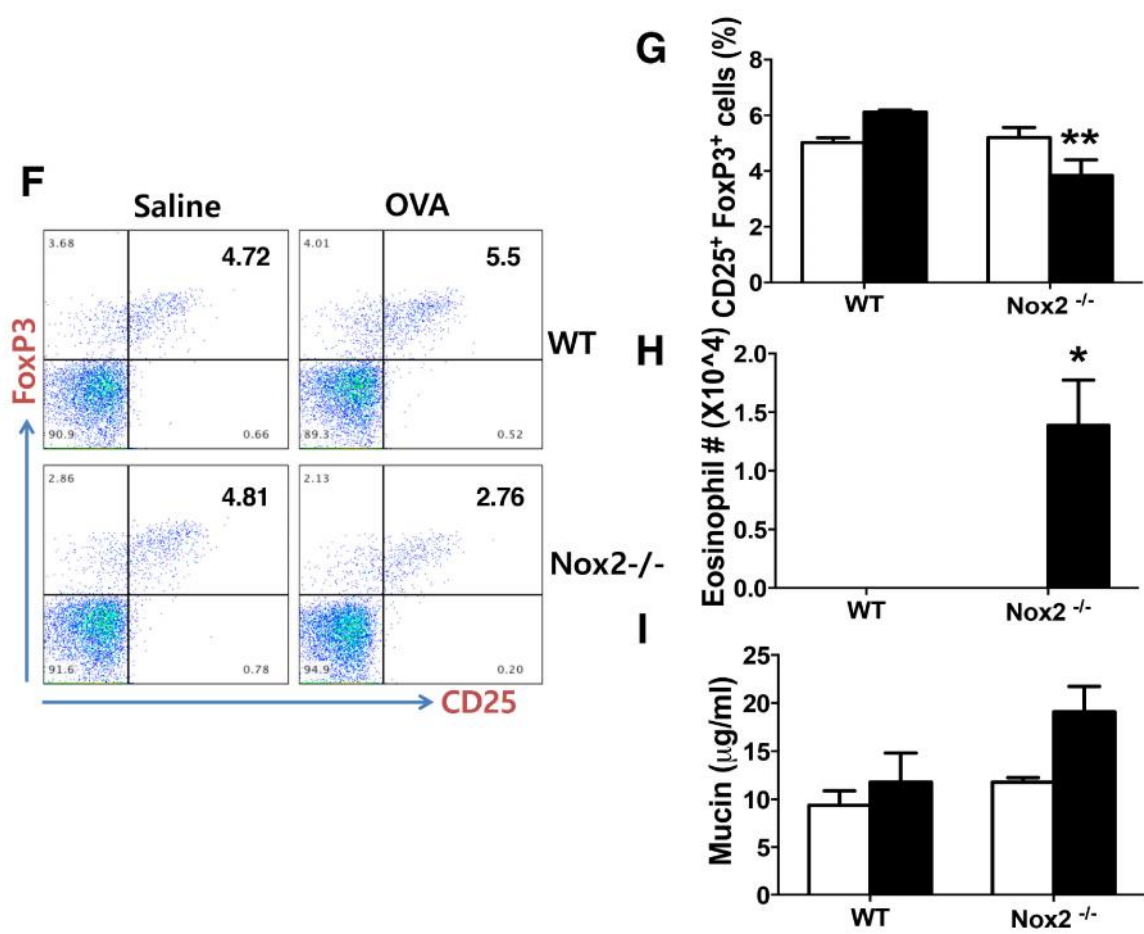
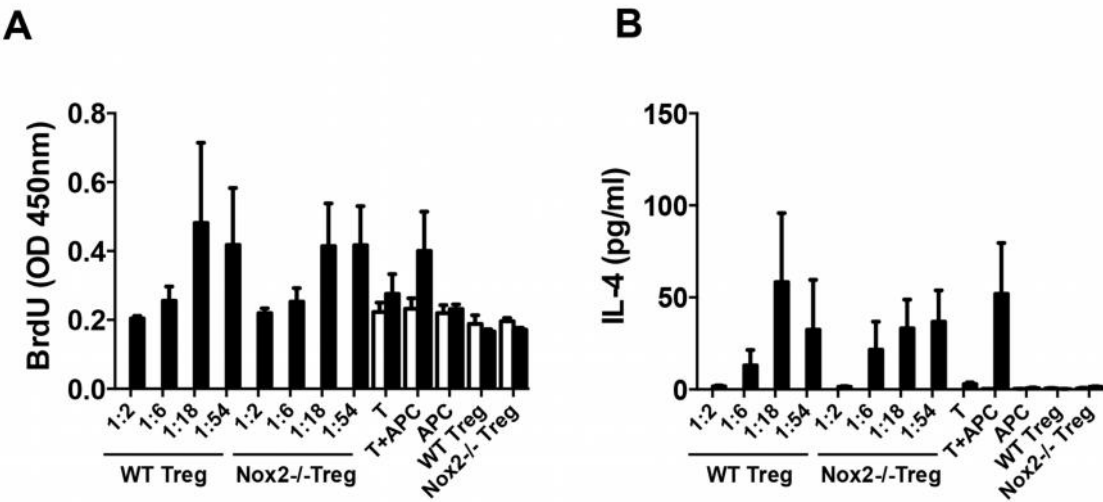


Figure S9



Supplemental Figure Legends

Figure S1 (relates to Figure 1) Exaggerated asthma symptoms in Nox2 deficient mice.

After allergic asthma was induced by intraperitoneal saline (□) or OVA/alum (■) and intranasal saline or OVA challenge, inflammatory cells (A), eosinophilia (B), glycoprotein secretion (C) in BAL fluid were measured, and IL-4⁺ cell numbers in lung analyzed by ELISpot (D). Data are presented as mean ± SEM, *** $p < 0.0005$ and * $p < 0.05$ compared with WT OVA challenged mice and representative of three independent experiments (n = 3).

Figure S2 (relates to Figure 2) Enhanced asthma phenotypes in Nox2 null mice.

Asthma was induced by intraperitoneal and intranasal OVA only challenge. AHR (A, ○: WT saline, □: Nox2^{-/-} saline, ●: WT OVA, ■: Nox2^{-/-} OVA, ▲: positive control (OVA/Alum and OVA)), eosinophilia (B), glycoprotein secretion (C) in BAL fluid, and lung pathology with PAS staining (D) were examined. Data are representative of three independent experiments (n = 3, □: saline, ■: OVA) and presented as mean ± SEM, ** $p < 0.005$ and * $p < 0.05$ compared with WT OVA challenge.

Figure S3 (relates to Figure 3) Augmented asthma phenotypes in Nox2 null CD4 T cell reconstituted mice.

WT CD45.1 mice were reconstituted with either WT or Nox2 deficient OVA peptide specific OT-II CD4 T cells and APO allergen was administered three times intranasally. AHR measurement (A, ○: No transfer saline, □: WT OT-II transfer saline, Δ: Nox2^{-/-} OT-II transfer saline, ●: No transfer APO, ■: WT OT-II APO, ▲: Nox2^{-/-} OT-II APO), eosinophilia (B), glycoprotein secretion (C) and IL-4 production (D) in BAL fluid were examined. Data are presented as mean ± SEM, ** $p < 0.005$, and * $p < 0.05$ compared with APO challenged CD45.1 WT mice reconstituted with WT OT-II T cells and

representative of three independent experiments (n = 6, □: saline, ■: APO).

Figure S4 (relates to Figure 4) Hyper-activation of naïve CD4 T cells without Nox2.

Cell proliferation (A) and IL-4 (B) production measured by CFSE dilution and ELISA following stimulation with indicated amount (µg/ml) of soluble anti-CD3 and CD28 antibodies (1 µg/ml of antibodies for CFSE dilution). Data are presented as mean ± SEM, *** $p < 0.0005$ and * $p < 0.05$ compared with WT CD4 T cells stimulated with antibodies and are representative of three independent experiments (n = 3, ○: WT, ●: Nox2^{-/-}).

Figure S5 (relates to Figure 4) Enhanced activation of OT-II T cells in the absence of Nox2.

Hyper-activation of naïve OT-II CD4 T cells in Nox2 deficiency with media (□) or 10 µM of OVA peptide (■) stimulation. Cell proliferation (A) and IL-4 production (B) were assessed by BrdU assay and ELISA. Data are representative of three independent experiments (n = 3) and presented as mean ± SEM, *** $p < 0.0005$ and ** $p < 0.005$ compared with WT OVA peptide stimulation.

Figure S6 (relates to Figure 5) Decreased cytosolic ROS production in Nox2 deficiency.

Assessment of cytosolic ROS stained with DCF-DA by flow cytometry. Compiling data (A) and representative data (B) are shown. Data are presented as mean ± SEM, *** $p < 0.0005$ compared with WT stimulated with antibodies and representative of three independent experiments (n = 3, □: WT, ■: Nox2^{-/-}).

Figure S7 (relates to Figure 7) Diminished AICD by mitochondria specific anti-oxidants

treatments in early phase.

Caspase-3⁺ CD4 T cells were analyzed with or without pretreatment of NAC, MitoQ, or rotenone by flow cytometry after 12 hours with antibody stimulation (n=3, □: Ab, ▨: Ab + NAC, ▩: Ab + MitoQ, ■: Ab + rotenone). Representative (A) and compiling data (B) are shown. Data are presented as mean ± SEM, **** $p < 0.0001$, *** $p < 0.0005$ and ** $p < 0.005$ compared with indicated group and are representative of three independent experiments.

Figure S8 (relates to Figure 7) Reduced Treg differentiation in the absence of Nox2.

(A-C) Naïve CD4 T cells were stimulated with anti-CD3 and CD28 antibodies for 5 days under Treg cell differentiation conditions, the levels of CD25⁺FoxP3⁺ Treg cells and IL-4 production were enumerated by flow cytometry and ELISA analyses. Compiling data (A) and representative data (B) of Treg differentiation and IL-4 production level (C) are shown (□: media, ▨: neutral condition, ■: Treg condition). (D, E) Naïve CD4 T cells were cultured in Treg polarizing condition for 5 days with or without MitoQ or rotenone. Representative data (D) and compiling data (E) are shown. (□: media, ▨: Neutral condition, ■: Treg condition, ▩: Treg + MitoQ ▩: Treg + rotenone). (F-I) Tolerance was induced by intranasal OVA challenge and lung Treg cells were assessed by flow cytometry. Representative (F) and compiling data (G) of Treg differentiation and eosinophilia (H) and glycoprotein secretion (I) in BAL fluid are shown (□: saline, ■: OVA). Data are representative of three independent experiments (n = 4) and are presented as mean ± SEM, *** $p < 0.0005$, ** $p < 0.005$ and * $p < 0.05$ compared with in vitro WT Treg cell differentiation (A-E) or in vivo (F-I) experiments.

Figure S9 (relates to Figure 7) Normal Treg cell function in the absence of Nox2.

(A) Proliferation assessed with BrdU incorporation assay after stimulation with indicated antibodies.

(B) Quantification of IL-4 production by ELISA. Data are presented as mean $\pm$ SEM, compared with responder cells co-cultured with WT Treg cell, and representative of three independent experiments (n = 3, $\square$: media, $\blacksquare$: antibody).

Additional Materials and Methods

Mice

CD45.1 and CD45.2 wild-type (WT) C57BL/6, C57BL/6 background recombinate activating gene-1 (Rag-1) deficient, Nox2 deficient, and T cell receptor transgenic OT-II mice were purchased from Jackson laboratories (Bar Harbor, ME). Six to eight week old female mice were used for this study. Animal care and experimental procedures were performed with the approval of the Animal Care Committee of KAIST.

Allergens and other reagents

Chicken egg ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) was precipitated in aluminum sulfate (OVA/alum) as described (1). *Aspergillus oryzae* protease (AP) allergen was purchased from Sigma-Aldrich and mixed with OVA. For ELISA assay, antibodies JES6-1A12, 11B11 and eBio13A were used for cytokine capture and biotinylated antibodies JES6-5H4, BVD6-24G2 and eBio1316H for detection of IL-2, IL-4 and IL-13 respectively were purchased from BD biosciences (San Diego, CA) except eBio13A and eBio1316H which are from eBioscience (San Diego, CA). For bronchoalveolar lavage (BAL) glycoprotein detection, biotinylated jacalin was purchased from Vector Laboratories (Burlingame, CA).

In vitro T cell stimulation

WT or Nox2 deficient naïve CD4 T cells (2×10^5) purified from splenocytes using naïve CD4 T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) were stimulated with 1 μ g/ml of anti-CD3 antibody (clone: 145-2C11) with or without anti-CD28 antibody (clone: 37.51) with or without 2 hour pretreatment of 0.1 μ g/ml MitoQ (MitoQ Ltd, Auckland, New Zealand), Rotenone

(Sigma-Aldrich), 30 μ M NAC (Sigma-Aldrich) and 1 μ M gp91ds-tat (AnaSpec Inc., Fremont, CA) in flat bottom 96-well cell culture plates or with irradiated (3,000 rads) T cell depleted splenocytes as an antigen presenting cells (APCs) in round bottom 96-well plate (BD bioscience). For anti-CD3 antibody stimulation, 3 fold serially diluted antibody (starting 1 μ g/ml until 1/27 μ g/ml) was used in flat bottom 96-well plate. After 4 days of culture, cell pellets were collected for cell proliferation analysis and supernatants were collected for cytokine assay. For T cell proliferation, either BrdU/MTT incorporation kit (Roche Applied Bioscience, Indianapolis, IN) or Carboxyfluorescein succinimidyl ester (CFSE) labeling (Life Technologies, Carlsbad, CA) was used according to the manufacturers' instruction. From original supernatants, the levels of IL-2 and IL-4 were quantified using standard sandwich ELISA as described (2).

Allergen administration and analysis of asthma phenotypes

Asthma induction was performed as described before (1, 3). Briefly, AP (1 mg/ml) and OVA (0.5 mg/ml) allergens were mixed 1:9 (v/v, APO allergen) immediately before administration. Mice were challenged with 50 μ L of APO allergen intranasally every 4 days for 5 times. To evaluate the therapeutic effects of MitoQ and Rotenone, MitoQ or Rotenone (500 μ M) was administered intratracheally 0.5h before APO challenge. For OVA only challenge, mice were either administered with 50 μ L of OVA intranasally every 4 days for 4 times, or sensitized with 50 μ L OVA intraperitoneally every 7 days for twice and beginning on day 14, the sensitized mice were administered 50 μ L OVA intranasally daily for 4 consecutive days. In other experiments, mice were sensitized with 200 μ L OVA/alum intraperitoneally every 7 days for twice and beginning on day 14, the mice were challenged with 50 μ L OVA intranasally for 4 days. Data were collected 16 hours after the final allergen challenge.

For all saline and allergen-challenged mice, BAL cytology, glycoprotein, and lung histopathology were determined as previously described (4). Airway hyper-responsiveness (AHR)

was measured with flexiVent system (SCIREQ Inc, Montreal, Canada). Briefly, the next day following final intranasal challenge, mice were intubated by using 20-gauge cannula and connected through intratracheal cannula to a flexiVent system, in an anesthesia state with pentobarbital sodium (60 mg/kg, Hanlim Pharma Co, Seoul, Korea). After paralysis with pancuronium bromide (1 mg/kg, Sigma-Aldrich), mice were ventilated at a respiratory rate of 150 breath/min and tidal volume of 10 ml/Kg against a positive end expiratory pressure (PEEP) of 3 CmH₂O. By administrating different doses of metacholine (0, 1, 3, 9, 18, 27 mg/ml, Sigma-Aldrich), airway resistance was measured.

Measurement of secreted glycoprotein in BAL fluid

Secreted glycoprotein in BAL fluid was quantified, as previously described (3). Briefly, glycoprotein mucin standard (derived from porcine stomach, Sigma-Aldrich) and BAL fluid samples were diluted in PBS serially 2-fold beginning at 1:100 dilution and 40 µL of them were transferred to flat bottom ELISA plate (Greiner, Kremsmunster, Austria) and incubated at 37°C for 2 hours. After washing, the plates were blocked by adding 200 µl of 0.2% I-block, and incubated at 37°C for 2 hours. After washing, 40 µL of biotin conjugated jacalin was added for detection and plates were incubated at 4°C overnight. After another washing, 40 µL of streptavidin-alkaline phosphatase diluted in PBS/Tween/BSA (1:1000 dilution) was added and the plates were incubated at room temperature for 30 minutes. After final washing, 70 µL of alkaline phosphatase substrate (5 mmole/L of p-nitrophenyl phosphate substrate in 0.1 mol/L alkaline buffer) was added and incubated for color development at room temperature. When color development was sufficient, the reaction was terminated by adding of 40 µL of 0.5N sodium hydroxide. Optical density was measured at 405 nm by an ELISA plate reader (BioRad, Hercules, CA).

Adoptive transfer of CD4 T cells

WT and Nox2 deficient CD4 T cells or OT-II CD4 T cells (5×10^6) were injected intravenously into either Rag-1 null or CD45.1 mice. Next day, mice were challenged with 50 μ L of APO allergen intranasally every 4 days for 3 times. Allergic lung inflammation data were collected 16 h after the final intranasal challenge.

Quantitation of cytokine-producing cells.

Total IL-4 and IL-13 producing cells were quantitated as previously described (4). Lung Th2 were determined by ELISpot assay from spontaneous cultured total CD4⁺ T cells.

Flow Cytometry

Single cell suspensions prepared from spleens and lungs of allergen challenged mice were incubated with the indicated fluorochrome-conjugated antibodies and isotype controls: Fluorescein Isothiocyanate (FITC) conjugated anti-CD4 (GK1.5); Phycoerythrin (PE)-Cy7 conjugated anti-CD4 (RM4-5); Pacific Blue conjugated anti-CD3 (500A2); Allophycocyanin (APC)-Cy7 conjugated anti-CD44 (IM7); PE conjugated anti-IL-4 (11B11); APC conjugated anti-FoxP3 (FJK-16s); PE conjugated anti-CD25 (PC61.5); PE conjugated anti-caspase-3 (C92-605); 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA); MitoSOXTM Red mitochondrial superoxide indicator; Carboxyfluorescein succinimidyl ester (CFSE) (all antibodies from BD biosciences except anti-FoxP3 and anti-CD25 antibodies which are from eBioscience, and DCFDA and MitoSOX from Invitrogen (Carlsbad, CA) and CFSE from Life Technologies). Data were collected using a LSRII flow cytometer (BD biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

In vitro and in vivo Treg differentiation

For in vitro Treg differentiation, WT or Nox2 deficient naïve CD4 T cells (2×10^6) were stimulated with anti-CD3 (5 μ g/ml) and anti-CD28 (1 μ g/ml) antibodies in flat bottom 96-well cell culture plates. For Treg condition, TGF- β 1 (5ng) was added and polarization was assessed by examining FoxP3 expression.

For in vivo Treg induction, WT or Nox2 null mice were challenged with 50 μ L of OVA intranasally every 4 days for 2 times. The level of Treg induction was measured by examining FoxP3 expression in CD4 T cells.

Protein extraction and western blot analysis

For western blot analysis, lungs and spleens were removed from saline- and allergen-challenged mice and lysed by RIPA buffer (Cell Signaling, Billerica, MA). The lysates were separated by a 10% SDS-PAGE, and transferred into the nitrocellulose membranes. After blocking with either 5% skim milk or 5% BSA for 1 hour, the membranes were incubated with anti-actin, anti-Akt, anti-phospho Akt (Thr308), anti-phospho Akt (Ser473) and anti-GATA3 (all antibodies from cell signaling except anti-actin which is from Sigma-Aldrich and anti-GATA3 from BD biosciences) overnight at 4°C, followed by incubating with HRP-conjugated secondary antibodies (Cell signaling) for 1 hour at room temperature. Signals were developed with West Save Up™ (AbFrontier, Seoul, Korea) and SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA), detected using LAS-4000 (Fujifilm, Tokyo, Japan), and quantified the intensities of bands by using Multi Gauge (Fujifilm).

Treg functional assay

WT and Nox2 deficient CD4⁺CD25^{hi} Treg cells and CD4⁺CD25⁻ responder WT T cells were stimulated with 1 µg/ml of soluble anti-CD3 and CD28 antibodies in U bottom 96-well plates. CD4⁺CD25⁻ responder T cells (5×10^4) were also cultured with decreasing number of CD4⁺CD25⁺ Treg cells (Treg to responder ratios: 1:2, 1:6, 1:18, and 1:54). T cell depleted splenocytes (10×10^4 /well) were used as APCs in a final volume 200 µl. After 4 days of culture, supernatant was removed from each well for cytokine analysis using standard ELISA. Cell pellets were collected for cell proliferation using BrdU incorporation assay.

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