

# Cigarette smoke inhibits ROCK2 activation in T cells and modulates IL-22 production



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## ABSTRACT

Gene-environment interactions are known to play a key role in the development of rheumatoid arthritis (RA). Exposure to cigarette smoke (CS) is one of the strongest environmental risk factors associated with RA and has been shown to mediate a range of complex immunomodulatory effects from decreased T and B cell activation to depressed phagocytic function. The effects of CS on the function of T<sub>H</sub>17 cells, one of the key T<sub>H</sub> effector subsets implicated in RA pathogenesis, are not fully understood. IRF4 is one of the crucial transcription factors involved in T<sub>H</sub>17 differentiation and is absolutely required for the production of IL-17 and IL-21 but, interestingly, inhibits the synthesis of IL-22. The production of IL-17 and IL-21 by IRF4 can be augmented by its phosphorylation by the serine-threonine kinase ROCK2. Given that CS has been reported to increase ROCK activity in endothelial cells, here we investigated the effects of CS on the ROCK2-IRF4 axis in T cells. Surprisingly, we found that CS leads to decreased ROCK2 activation and IRF4 phosphorylation in T cells. This effect was associated with increased IL-22 production. Using a GEF pull-down assay we furthermore identify ARHGEF1 as a key upstream regulator of ROCK2 whose activity in T cells is inhibited by CS. Thus CS can inhibit the ROCK2-IRF4 axis and modulate T cell production of IL-22.

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## 1. Introduction

Rheumatoid arthritis (RA) is characterized by the infiltration of immune cells into the synovium eventually resulting in cartilage destruction and bone erosions (McInnes and Schett, 2011). The development of RA is mediated through a complex interaction between environmental and genetic factors (Costenbader et al., 2012; Gerlag and Norris Tak, 2015). Amongst environmental risk factors, cigarette smoke (CS) exposure has been strongly associated with the development of RA (Arnson et al., 2010; Hoovestol and Mikuls 2011; Klareskog et al., 2011). CS has been shown to exert a number of complex immunomodulatory effects from decreased T and B cell activation to depressed phagocytic function to increased

oxidative stress (Baka and Buzas Nagy, 2009). In line with the broad and multifaceted effects of CS on immune responses, exposure of mice to cigarette smoke has been reported to either augment or delay collagen-induced arthritis (CIA), with the latter effect being associated with lower autoantibody responses (Lindblad et al., 2009; Chujo et al., 2010; Okamoto et al., 2011).

CD4<sup>+</sup> T helper cells play a key role in the pathogenesis of many autoimmune diseases, including RA. In particular, one of the T<sub>H</sub> effector subsets, the T<sub>H</sub>17 subset has been implicated in the development of RA via its ability to produce key cytokines such as IL-17, IL-21, and IL-22 (Koenders and van den Berg 2015; Lubberts 2015). Aberrant production of IL-17 and IL-21 has been observed in murine models of RA and in patients affected by this disorder and blockade of IL-17- and IL-21-mediated responses has been found to be efficacious in ameliorating disease in murine models of RA (Pernis 2009). Higher expression levels of IL-22, a member of the IL-10 cytokine family, have also been observed in synovium from RA patients as

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well as in mice with CIA (Rutz, et al., 2013; Yang and Zheng, 2014; Xie et al., 2015).

Critical to  $T_H$ -17 differentiation is a transcription factor, Interferon Regulatory Factor 4 (IRF4), which is absolutely required for IL-17 and IL-21 production (Brustle et al., 2007; Chen et al., 2008; Huber et al., 2008). Interestingly, while IRF4 promotes the production of IL-17 and IL-21, it inhibits the synthesis of IL-22 (Valdez et al., 2012). During a search for proteins interacting with IRF4, our laboratory isolated a novel protein termed Def6 (also known as IBP or SLAT) (Hotfilder et al., 1999; Gupta et al., 2003; Tanaka et al., 2003). DEF6 serves a crucial immunoregulatory role *in vivo* as shown by the fact that Def6-deficient mice crossed to a TCR transgenic mouse (DO11.10) spontaneously develop RA-like disease due to enhanced IRF4 activation and dysregulated IL-17 and IL-21 production (Chen et al., 2008). One of the key mechanisms by which DEF6 regulates IRF4 function is by inhibiting its ability to be phosphorylated by ROCK2 (Biswas et al., 2010). The ROCK2-mediated phosphorylation of IRF4, indeed, increases its binding to the IL-17 and IL-21 promoters and leads to higher levels of IL-17 and IL-21 production (Biswas et al., 2010). ROCK2 and its other isoform, ROCK1, are serine-threonine kinases, which normally become activated upon binding of active GTP-bound RhoA (Amano et al., 2010; Schofield and Bernard, 2013; Thumkeo et al., 2013; Julian and Olson 2014). Aberrant ROCK activation has been implicated in the pathogenesis of cardiovascular, renal, and neurological disorders (Mueller et al., 2005; Zhou et al., 2011; Komers 2013). Interestingly, smoking has been shown to activate RhoA and the ROCKs in non-hematopoietic cells (Chiba et al., 2005; Noma, et al., 2005, 2007) and smokers exhibit increased leukocyte ROCK activity (Hidaka et al., 2010).

In view of the potential link between CS and the ROCKs here we explored whether CS could alter the ROCK2-IRF4 axis in T cells. To accomplish this goal we took advantage of the Def6<sup>-/-</sup> DO11.10 cell culture system because of the higher levels of activation of the ROCK2-IRF4 pathway in these T cells, which could facilitate the detection of any changes upon CSE exposure. Interestingly, exposure of T cells from Def6<sup>-/-</sup> DO11.10 mice to cigarette smoke extract (CSE) resulted in decreased activation of ROCK2 and lower levels of IRF4 phosphorylation. These effects were associated with increased synthesis of IL-22. Using a GEF pull-down assay we furthermore identify ARHGEF1 as a key upstream regulator of ROCK2 whose activity is inhibited by CSE. Thus CS can directly alter  $T_H$ -17 function via effects on the ROCK2-IRF4 axis.

## 2. Materials and methods

### 2.1. Mice

C57BL/6 were obtained from Jackson Laboratory. Mice with DEF6 deficiency were generated by Lexicon Pharmaceuticals (Omnibank) using gene-trapping strategy and hence were originally termed Def6<sup>trap/trap</sup> mice (Fanzo et al., 2006). The original mice on a mixed 129XC57BL/6 background were backcrossed to Balb/c mice for >10 generations. Def6<sup>trap/trap</sup> mice on a Balb/c background were then crossed to DO11.10 TCR transgenic mice (Jackson Laboratory) to generate Def6<sup>trap/trap</sup>DO11.10 mice. To simplify the terminology, these mice will be referred to as Def6<sup>+/+</sup>DO11.10 and Def6<sup>-/-</sup>DO11.10 mice in this manuscript. All mice used in the experiments were kept under specific pathogen-free conditions. Female mice (age 5–7 weeks old) were used in all experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery.

### 2.2. CD4<sup>+</sup> T cell isolation and cytokine production

CD4<sup>+</sup> T cells were purified from the spleens using CD4<sup>+</sup> T cell isolation kit from Miltenyi Biotech. For cytokine analysis,  $1 \times 10^6$  cells/ml were stimulated with plate-bound anti-CD3 $\epsilon$  (2  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml) for 3 days and then rested in IL-2 (20  $\mu$ g/ml) for 4 days. At day 7, cells were restimulated with plate-bound anti-CD3 $\epsilon$  (2  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml) for 24–48 h as previously described (Chen et al., 2008). Supernatants were analyzed for IL-17A (Biolegend), IL-21 (eBioscience), and IL-22 (Biolegend) production by ELISA.

### 2.3. Cell extracts and Western blotting

Nuclear and cytoplasmic extracts were prepared using the NEPER Nuclear and Cytoplasmic extraction reagent kit as previously described (Chen et al., 2008; Biswas et al., 2010). Anti-mouse ROCK1, ROCK2, IRF4 and ARHGEF1 Abs (Santa Cruz Biotechnology) were used to probe the Western blots according to the manufacturer's instructions. Rabbit polyclonal Ab specific for phospho-IRF4 was generated by 21st Century Biochemicals Inc., using a synthetic phosphopeptide (YHRSIRH[pS][pS]IQE) corresponding to amino acids 439–450 of human IRF4 as an immunogen (the amino acid numbering in IRF4 sequence is based on GenBank accession no. U52682) and was previously described (Biswas et al., 2010).

### 2.4. Real-time RT-PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen GmbH). cDNAs were prepared and analyzed for expression of the gene of interest by quantitative real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems) using a Sybr-Green PCR master mix kit. PCR primers used for *Il17*, *Il21*, *Il22*, *Irf4*, and *Roryt* have been previously described (Chen et al., 2008; Valdez et al., 2012). Primers for mouse *Arhgef1* were purchased from QIAGEN (Cat. QT02260727). The relative mRNA expression level of each gene was based on the comparison of the  $\delta$ Ct value of restimulated DO11.10 Th0 sample normalized to  $\beta$ -actin.

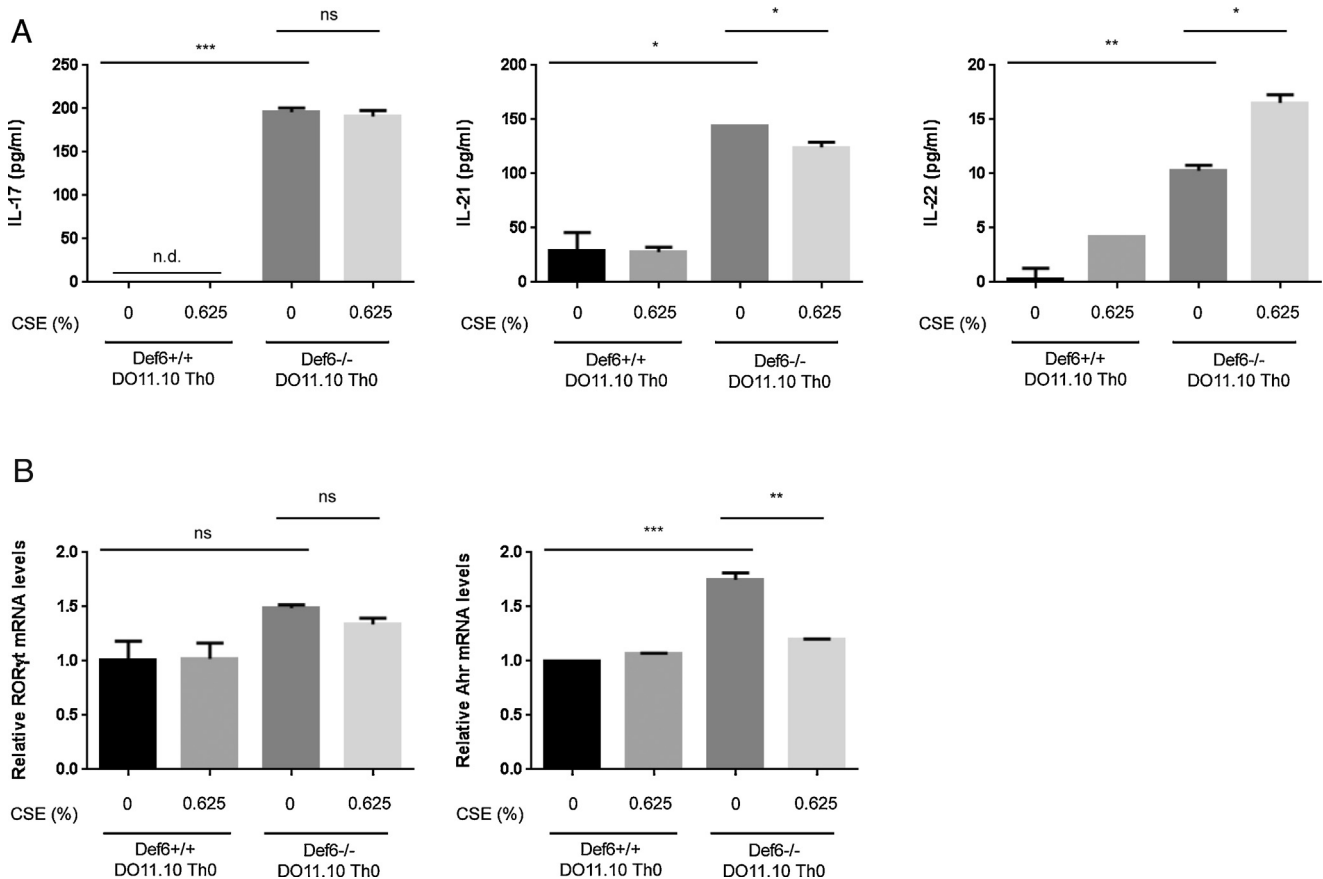
### 2.5. ROCK1/2 kinase activity assays

#### 2.5.1. ELISA

ROCK kinase activity in cell extracts was measured using the 96-well ROCK Activity Assay Kit (Cell Biolabs, Inc.) as previously described (Isgro et al., 2013). Briefly, whole cell extracts (WCE) were prepared using 1% NP-40 lysis buffer. 10  $\mu$ g of the WCE were then incubated in a 96-well plate pre-coated with MYPT1, a ROCK substrate, in the presence of kinase reaction buffer containing ATP. After extensive washing, the wells were incubated with anti-phospho-MYPT1 (Thr<sup>696</sup>) antibody. After 1-h, the wells were extensively washed and an HRP-conjugated secondary antibody was added for another hour. Substrate solution was then added to the wells and quantification performed on a spectrophotometer using 450 nm as the primary wavelength. Active ROCK2 (1–4 ng) served as a positive control as per the manufacturer's instructions.

#### 2.6. ROCK1 and ROCK2 kinase activity assays

For these assays, which utilize exogenous recombinant MYPT1 as the ROCK substrate (Mong and Wang, 2009), ROCK1 or ROCK2 were first immunoprecipitated from whole cell extracts of purified CD4<sup>+</sup> T cells using an anti-ROCK1 or anti-ROCK2 antibody (Santa Cruz Biotechnology). The immunoprecipitated ROCK1 or ROCK2 was then subjected to an *in vitro* kinase reaction by incubating with purified recombinant MYPT1 substrate added exogenously in kinase buffer containing ATP according to the manufacturer's



**Fig. 1.** Effect of cigarette smoke extract (CSE) on cytokine production by *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells from either *Def6*<sup>+/+</sup> DO11.10 or *Def6*<sup>-/-</sup> DO11.10 mice were purified and cultured under neutral (Th0) ( $\alpha$ CD3 2  $\mu$ g/ml,  $\alpha$ CD28 1  $\mu$ g/ml) conditions for 3 days and then rested for 4 days in IL-2 (20 ng/ml). After resting, CD4<sup>+</sup> T cells were restimulated under neutral (Th0) conditions in the presence or absence of sterile CSE (0.625%) for 24 h. (A) Cytokine levels of IL-17, IL-21, and IL-22 in culture supernatants were quantified by ELISA; n.d., cytokine levels non-detectable. (B) mRNA expression levels of ROR $\gamma$ t and AHR were measured by quantitative real-time RT-PCR. Data are representatives of three independent experiments. Statistical analyses were performed by unpaired Student's *t* test; ns,  $P \geq 0.05$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

instructions (Cell Biolabs). Phosphorylation of recombinant MYPT1 substrate by immunoprecipitated ROCK1 or ROCK2 was detected by Western blotting using an antiphospho-MYPT1 (T696) antibody.

### 2.7. Active ARHGEF1 pull-down

RhoA-G17A-conjugated agarose beads (Cell Biolabs; Cat. STA-431) were used to pull-down active ARHGEF1 from the whole cell extracts prepared from each condition, following the manufacturer's instructions. Precipitated active ARHGEF1 was detected by Western blotting using an anti-ARHGEF1 antibody.

### 2.8. Cigarette smoke extract

Cigarette smoke extract (CSE) was prepared by bubbling the smoke of one cigarette (3R4F, University of Kentucky, Lexington, KY) through 25 ml of PBS for ten minutes. The cigarette smoke extract was pH balanced to 7.4 and sterile filtered prior to use (Mehra et al., 2012). We primarily used 0.625% CSE, which equates to  $\approx$ 5 cigarettes per day (Cawood et al., 2007).

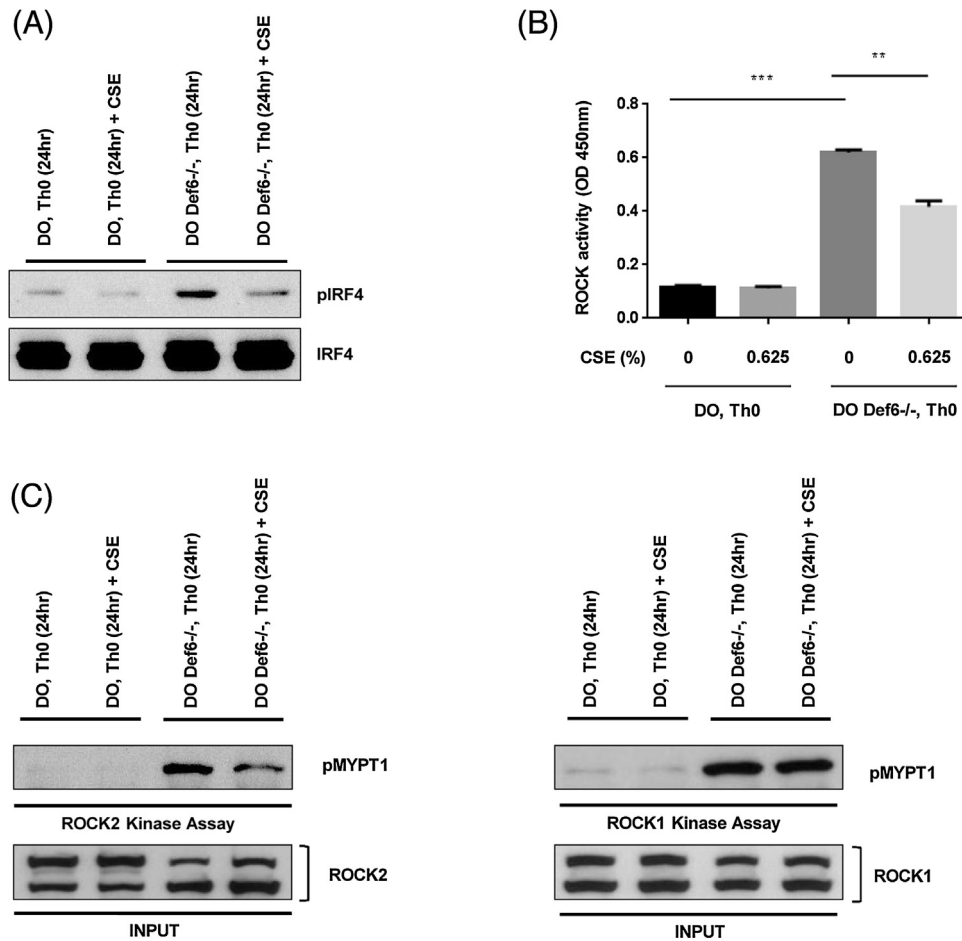
### 2.9. Statistical analysis

Two-tailed Student's *t* test was applied to all our *in vitro* studies. The statistical differences were considered significant when  $p$ -value  $< 0.05$ . Data were presented as mean  $\pm$  SEM.

## 3. Results

### 3.1. Exposure of autoimmune CD4<sup>+</sup> T cells to CSE induces the production of interleukin-22 (IL-22) under neutral conditions

CS has previously been shown to increase ROCK activation in non-hematopoietic cells (Chiba et al., 2005; Noma et al., 2005; Noma et al., 2007). In view of the ability of the RhoA-ROCK pathway to promote the production of IL-17 and IL-21 by autoimmune T cells we proceeded to investigate whether CSE could further modulate the production of these cytokines. To gain insights into this question, we employed *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells, which exhibit dysregulated ROCK2 activity and aberrant production of IL-17 and IL-21 under neutral conditions (Biswas et al., 2010). CD4<sup>+</sup> T cells were purified and expanded *in vitro* under neutral conditions and then restimulated for 24 h, culture conditions that have previously been shown to result in aberrant IL-17 and IL-21 production by *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells as compared to control *Def6*<sup>+/+</sup> DO11.10CD4<sup>+</sup> T cells (Chen et al., 2008). Pilot experiments demonstrated that addition of CSE at a concentration of  $>1.25\%$  resulted in significant toxicity leading us to employ a CSE concentration of 0.625%, which was accompanied by no or minimal toxicity (data not shown). CSE was added upon restimulation of the cells and cytokine production in the supernatants assessed by ELISA. As compared to control CD4<sup>+</sup> T cells, *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells exhibited increased IL-17 and IL-21 production, which was not affected by exposure to CSE in a consistent manner (Fig. 1A). In line with these results, CSE did not alter the increased expression of



**Fig. 2.** Exposure of *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells to cigarette smoke extract (CSE) inhibits IRF4 phosphorylation and ROCK2 kinase activity. CD4<sup>+</sup> T cells from either *Def6*<sup>+/+</sup> DO11.10 (DO Th0) or *Def6*<sup>-/-</sup> DO11.10 (DO Def6<sup>-/-</sup> Th0) mice were purified and cultured under neutral (Th0) ( $\alpha$ CD3 2  $\mu$ g/ml,  $\alpha$ CD28 1  $\mu$ g/ml) conditions for 3 days and then rested for 4 days in IL-2 (20 ng/ml). After resting, CD4<sup>+</sup> T cells were restimulated under neutral (Th0) conditions in the presence or absence of sterile CSE (0.625%) for 24 h. (A) Nuclear extracts from each condition were assayed for IRF4 phosphorylation by Western blotting using an antibody directed against phosphorylated IRF4 (pIRF4) (upper panel). Total IRF4 levels were evaluated by reprobing with an antibody against total IRF4 (lower panel). (B) Total ROCK kinase activity in whole cell extracts from each condition was assessed by an ELISA-based assay. (C) ROCK2 (left) and ROCK1 (right) kinase activity was examined by incubating immunoprecipitated ROCK2 or ROCK1 with purified recombinant MYPT1 (rMYPT1) as substrate. Phosphorylated rMYPT1 (pMYPT1) (shown in upper panel) was then detected using anti-phospho-MYPT1 antibody. Total ROCK2 or ROCK1 levels for each sample are shown in the lower panel. Data are representatives of two independent experiments. Statistical analyses were performed by unpaired Student's *t* test; ns,  $P \geq 0.05$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

ROR $\gamma$ t, a key transcription factor controlling the production of IL-17 (Fig. 1B). Interestingly, however, we reliably observed that CSE upregulated IL-22 production (Fig. 1A). This effect was not due to increased expression of AHR, a transcription factor known to regulate IL-22 production (Fig. 1B). Exposure to CSE can thus lead to the upregulation of IL-22 production by autoimmune T cells.

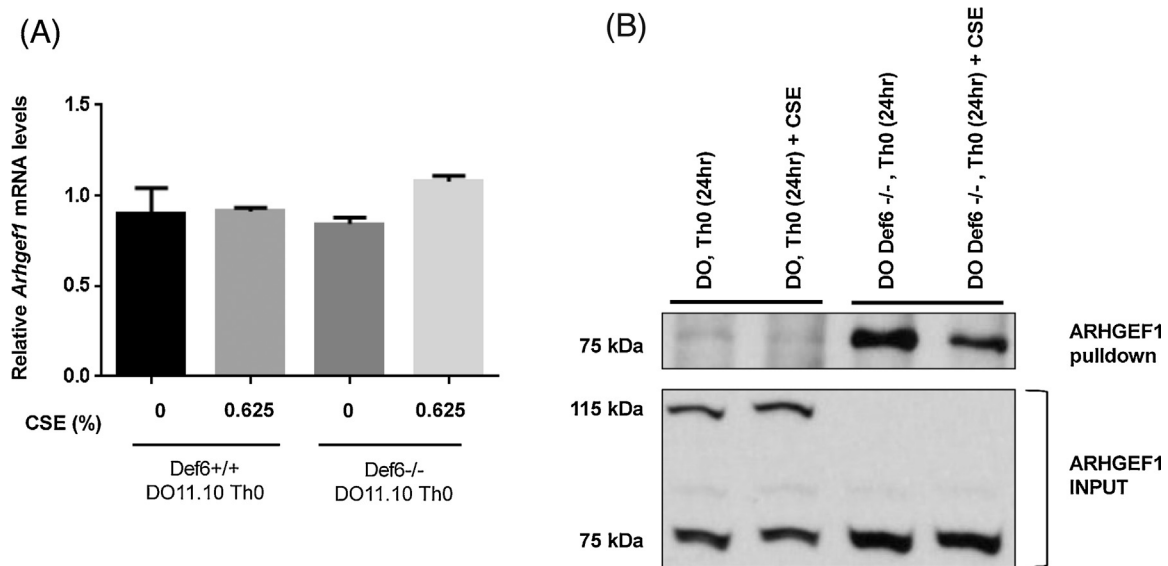
### 3.2. CSE exposure results in reduced IRF4 phosphorylation and ROCK2 kinase activity in *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells

The increased production of IL-22 upon CSE exposure was surprising given that IRF4 normally inhibits IL-22 production and that *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells are known to exhibit enhanced IRF4 activity due to increased phosphorylation by ROCK2 (Biswas et al., 2010; Valdez et al., 2012). We thus proceeded to directly evaluate the effects of CSE on the ROCK2–pIRF4 axis. Interestingly, CSE-treated *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells exhibited lower levels of phosphorylated IRF4 compared to unexposed samples, although CSE did not significantly affect total IRF4 levels (Fig. 2A). In line with these findings a ROCK ELISA assay, which measures total ROCK activity (encompassing both ROCK1 and ROCK2) demonstrated lower levels of ROCK kinase activity (Fig. 2B). To directly

assess whether ROCK2 rather than ROCK1 was affected by CSE we immunoprecipitated ROCK1 or ROCK2 and directly tested their kinase activity by incubating the precipitates with MYPT1. Exposure to CSE inhibited ROCK2 kinase activity to a much greater extent than ROCK1 activity (Fig. 2C). Thus, CSE selectively reduces ROCK2 kinase activity resulting in decreased IRF4 phosphorylation.

### 3.3. CSE reduces activation of ARHGEF1, an upstream regulator of the RhoA-ROCK pathway

ROCK activation is primarily regulated upon binding of activated (or GTP-bound) RhoA to its RhoA Binding Domain (RBD). GTP loading of RhoA is, in turn, controlled by the activation of Rho guanine nucleotide exchange factors (GEFs) (Amano et al., 2010; Schofield and Bernard, 2013; Thumkeo et al., 2013; Julian and Olson 2014). While a number of RhoGEFs exist within a cell, ARHGEF1 is known to be a major regulator of RhoA activation (Hart et al., 1998; Kozasa et al., 1998; Guan et al., 2013; Cook et al., 2014). Real-time PCR analysis demonstrated that ARHGEF1 is expressed in *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells at levels equivalent to *Def6*<sup>+/+</sup> DO11.10CD4<sup>+</sup> T cells and that its mRNA expression is not affected by exposure to CSE (Fig. 3A). To investigate whether the decreased activity of



**Fig. 3.** CSE decreases the activity of ARHGEF1 in *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells from either *Def6*<sup>+/+</sup> DO11.10 (DO, Th0) or *Def6*<sup>-/-</sup> DO11.10 (DO *Def6*<sup>-/-</sup>, Th0) mice were purified and cultured under neutral (Th0) ( $\alpha$ CD3 2  $\mu$ g/ml,  $\alpha$ CD28 1  $\mu$ g/ml) conditions for 3 days and then rested for 4 days in IL-2 (20 ng/ml). After resting, CD4<sup>+</sup> T cells were restimulated under neutral (Th0) conditions in the presence or absence of sterile CSE (0.625%) for 24 h. (A) mRNA expression levels of *Arhgef1* were measured by quantitative real-time RT-PCR. Statistical analyses were performed by unpaired Student's *t* test; ns,  $P \geq 0.05$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . (B) RhoA G17A-conjugated agarose beads were used to pull-down active ARHGEF1 from whole cell extracts of CD4<sup>+</sup> T cells prepared from each condition. Precipitated active ARHGEF1 (upper panel) and total ARHGEF1 (input) levels in each sample (lower panel) were detected by Western blotting using an antibody against ARHGEF1. Data are representatives of two independent experiments.

ROCK2 could be accounted for by changes in the activity level of ARHGEF1, we next employed an active Rho-GEF pull-down assay (Arthur et al., 2002; Garcia-Mata et al., 2006). In this assay, agarose beads coupled with a nucleotide free form of RhoA (G17A), which stabilizes the interaction between RhoA and the activated RhoGEF, are incubated with cell extracts and the presence of active ARHGEF1 visualized by western blotting with an ARHGEF1 antibody. Interestingly, while *Def6*<sup>+/+</sup> DO11.10CD4<sup>+</sup> T cells expressed both a 100 kDa and a 75 kDa isoform of ARHGEF1, *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells expressed primarily the shorter 75 kDa isoform (Fig. 3B). Consistent with the higher levels of ROCK2 activity in *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells, ARHGEF1 activity was higher in *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells than in *Def6*<sup>+/+</sup> DO11.10CD4<sup>+</sup> T cells (Fig. 3B). Importantly, exposure to CSE substantially diminished the activity of ARHGEF1 in *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells (Fig. 3B). Thus, *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells exhibit increased levels of ARHGEF1 activity suggesting that this is the key regulator of ROCK2 activation in these cells. The inhibitory effects of CSE on ARHGEF1 activity, but not on its mRNA or protein expression, furthermore, suggest that the capacity of CSE to decrease ROCK2 activation in these cells relies on its ability to interfere with ARHGEF1 activation.

#### 3.4. Exposure to CSE upregulates IL-22 production in T<sub>H</sub>-17 cells from non-autoimmune mice

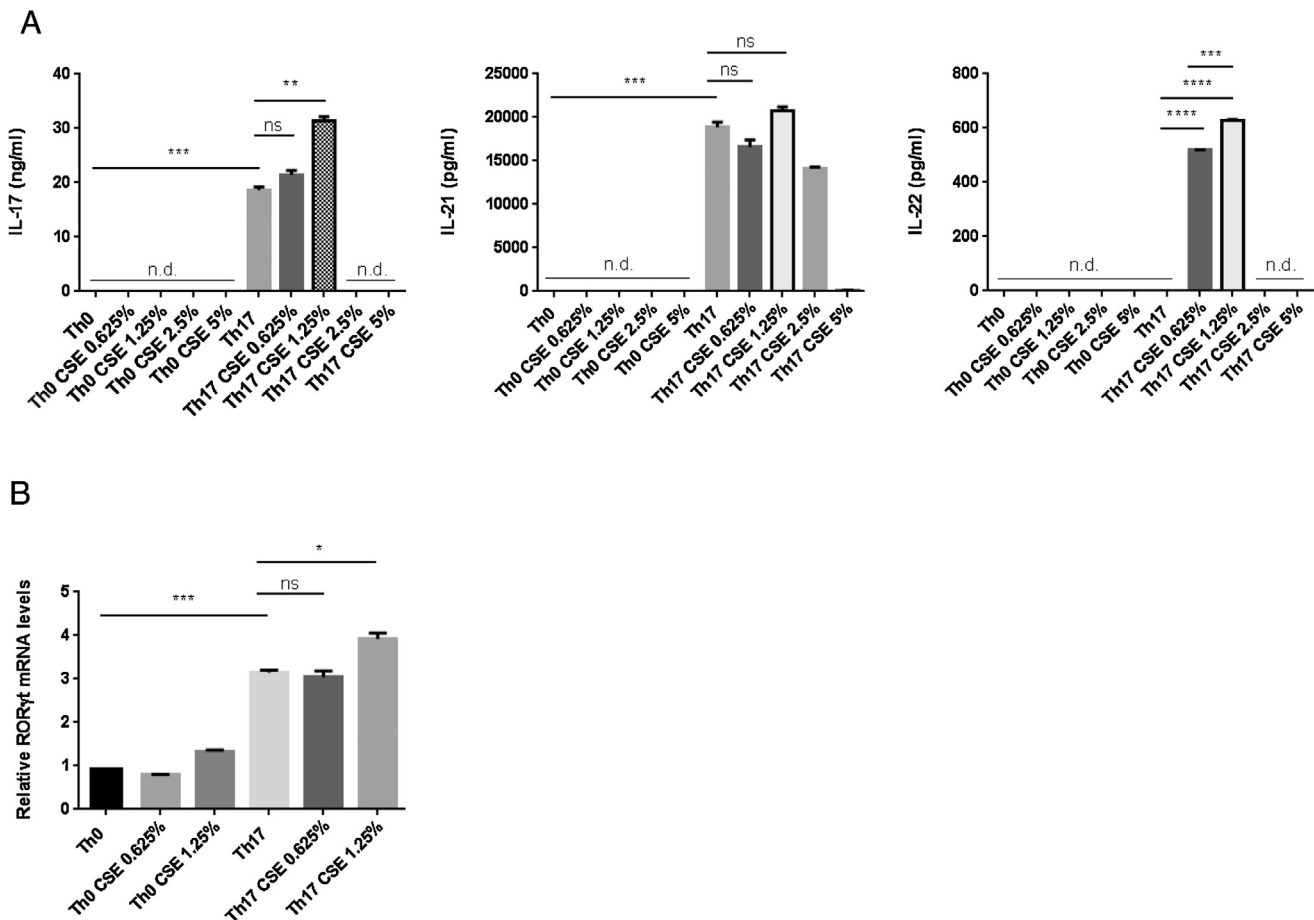
Unlike what is observed with *Def6*<sup>-/-</sup> DO11.10CD4<sup>+</sup> autoimmune T cells, production of IL-22 by nonautoimmune T cells requires exposure to specific cytokine milieu such as those encountered upon T<sub>H</sub>-17 differentiation (Rutz et al., 2013). To evaluate whether the effects of CSE on IL-22 production could also be observed in differentiating T<sub>H</sub>-17 cells, we sorted naïve T cells from C57BL/6 mice and differentiated them under neutral (T<sub>H</sub>0) or T<sub>H</sub>-17 conditions for 3 days in the presence/absence of increasing doses of CSE (Fig. 4A). Similar to the DO11.10 T cell system, CSE doses  $\geq 1.25\%$  exerted profound inhibitory effects on cytokine production due to its toxic effects on T cells. While the effects of lower doses (0.625% and 1.25%) of CSE on IL-17 and IL-21 production were again variable,

addition of CSE at those doses consistently upregulated IL-22 production by T<sub>H</sub>-17 cells (Fig. 4A). No effects of CSE could instead be detected in T<sub>H</sub>0 cells (Fig. 4A). The upregulation of IL-22 by 0.625% CSE furthermore was not accompanied by changes in the expression of ROR $\gamma$ t in cells cultured under T<sub>H</sub>-17-polarizing condition (Fig. 4B). Exposure to low-levels of CSE thus leads to the upregulation of IL-22 production by non-autoimmune T cells exposed to T<sub>H</sub>-17 differentiating condition.

#### 4. Discussion

Given the pathogenic role of T<sub>H</sub>-17 cells in RA and the strong association between CS and the development of RA here we evaluated whether CS exerts direct effects on the ability of CD4<sup>+</sup> T cells from either autoimmune or nonautoimmune mice to produce T<sub>H</sub>-17 cytokines. We found that exposure to CS consistently resulted in upregulation of IL-22 production. This was associated with the ability of CSE to downregulate ROCK2 activation and the phosphorylation of IRF4, a known negative regulator of IL-22 production (Valdez et al., 2012). Interestingly, the inhibitory effects of CSE on ROCK2 activity were accompanied by a decrease in the activation of ARHGEF1, an upstream regulator of RhoA. Thus CS can alter T cell function by modulating the activity of the RhoA-ROCK2 pathway.

The ability of CSE to increase IL-22 production could provide a potential new mechanism by which CS can promote the development of RA. Indeed increased IL-22 production has been observed in peripheral blood mononuclear cells and joints of RA patients and serum levels of IL-22 have been associated with disease severity (Rutz et al., 2013; Yang and Zheng, 2014; Xie et al., 2015). Furthermore, IL-22 can mediate a number of effects that have been linked to RA pathogenesis including driving the production of pro-inflammatory cytokines like IL-1 $\beta$ , IL-6, and TNF $\alpha$ , increasing expression of matrix metalloproteinase (MMP)-9, and promoting osteoclastogenesis. In line with these findings mice deficient in IL-22 exhibit delayed onset of CIA and decreased disease severity (Geboes et al., 2009). Intriguingly, the capacity of CS to drive IL-22 production may also underlie the known beneficial effects of smok-



**Fig. 4.** Effects of cigarette smoke extract (CSE) on cytokine production by nonautoimmune CD4<sup>+</sup> T cells cultured under either Th0 or Th17 conditions. FACS-sorted naïve CD4<sup>+</sup> T cells (CD4<sup>+</sup>, CD62L<sup>+</sup>, CD44<sup>-</sup>, CD25<sup>-</sup>) from wild-type C57BL/6 female mice were cultured under either neutral conditions (Th0) ( $\alpha$ CD3 2  $\mu$ g/ml,  $\alpha$ CD28 1  $\mu$ g/ml) or Th17 polarizing conditions ( $\alpha$ CD3 2  $\mu$ g/ml,  $\alpha$ CD28 1  $\mu$ g/ml, IL-6 20 ng/ml, TGF $\beta$  2 ng/ml) for 4 days in the presence or absence of the indicated dose of sterile cigarette smoke extract (CSE). (A) Cytokine levels of IL-17A (left panel), IL-21 (middle panel), and IL-22 (right panel) in the culture supernatants were quantified by ELISA; n.d., cytokine level non-detectable. (B) mRNA expression levels of ROR $\gamma$ t was measured by quantitative real-time RT-PCR. Statistical analyses were performed by unpaired Student's *t* test; ns,  $P \geq 0.05$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ . Data are representatives of three independent experiments.

ing in inflammatory bowel disease (Motley et al., 1987), where the antimicrobial and tissue regenerative functions of IL-22 may exert protective rather than pathogenic functions.

Unlike the consistent effects that CSE exerted on IL-22 production, its ability to modulate the synthesis of IL-17 and IL-21 was more variable possibly due to the complexities underlying the regulation of these different cytokines in DO11.10CD4<sup>+</sup> T cells. Indeed, despite the employment of some shared transcriptional modules, important differences exist in the control of IL-17 and IL-22 production (Rutz et al., 2013). Indeed activation of STAT3 is critical for the production of IL-22 but does not promote IL-17 production by itself. In contrast, although the T<sub>H</sub>-17 master regulator- ROR $\gamma$ t- is a positive regulator of both IL-17 and IL-22 expression, ROR $\gamma$ t alone can drive the expression of IL-17 but may regulate IL-22 indirectly. The requirement for the aryl hydrocarbon receptor (AHR) in the control of IL-22 production also differs depending on the presence or absence of T<sub>H</sub>-17 promoting cytokines like TGF $\beta$  whereby ligand-dependent activation of AHR is required to overcome the effects of c-Maf, which is induced downstream of TGF $\beta$  signaling and inhibits the production of IL-22 but promotes IL-17 synthesis.

Similarly to c-Maf, IRF4 is an activator of IL-17 and IL-21 but a repressor of IL-22 production. The simultaneous ability of CSE to downregulate IRF4 phosphorylation and upregulate IL-22 production thus suggests that the inhibitory effects of IRF4 may be more sensitive to its phosphorylation levels than its activating functions. Alternatively, distinct kinetics may be at play whereby the effects

of CSE on IRF4 phosphorylation, which require a series of inhibitory events starting with a decrease in the activation of ARHGEF1, may not take place in sufficient time to interfere with the ability of IRF4 to drive the production of IL-17 and IL-21. Given the known ability of CSE to mediate the activation of AHR (Esakky et al., 2012) it is also possible that CSE induces IL-22 production via a dual mechanism whereby it concomitantly promotes the activity of a transcriptional activator (AHR) while interfering with that of an inhibitor (IRF4). This dual mechanism may furthermore explain the different effects of CSE on IL-17 production in T<sub>H</sub>-17 cells versus the Def6<sup>-/-</sup> DO11.10CD4<sup>+</sup> T cells since AHR would be expressed at high levels in the T<sub>H</sub>-17 cultures but not in the Def6<sup>-/-</sup> DO11.10 cultures due to the presence of TGF- $\beta$  in the former but not the latter setting.

One of the most interesting aspects of our studies was the finding that the inhibitory effects of CSE on the ROCK2-IRF4 axis were associated with its ability to decrease the activation of a known upstream regulator of the RhoA-ROCK pathway, ARHGEF1. CSE is a complex mixture of substances including acrolein, nicotine, methyl vinyl ketone and volatile reactive oxygen species (Takanami et al., 2009; Noya et al., 2013). The precise component that diminished ARHGEF1 activity in T cells remains to be determined. However, it is important to note that oxidants, which are present in CSE, can alter the ability of guanine nucleotide exchange factors to interact with their targets (Wey et al., 2014). Thus, oxidative protein modifications could potentially explain why ARHGEF1 bound RhoA less avidly in the CSE treated T cells. While no effects of

CSE on the expression of ARHGEF1 were observed, we did detect different ARHGEF1 isoforms in wt versus Def6-deficient T cells. Since ARHGEF1 is known to be a 27-exon gene with multiple splice variants whose precise functions have not been defined (Muppidi et al., 2014), it will also be important in future studies to delineate whether these ARHGEF1 isoforms are differentially susceptible to the effects of CSE. Intriguingly, previous studies have shown that ARHGEF1-deficient mice spontaneously develop pulmonary pathology characterized by key features of emphysema with increased expression and activity of MMPs, airspace enlargement, and decreased lung elastance (Hartney et al., 2010). Of additional interest was the finding that macrophages lacking ARHGEF1 produce increased levels of MMP-2, -9 and -12 upon adhering to fibronectin (Hartney et al., 2011) suggesting that ARHGEF1 is critical for the ability of immune cells to properly sense cues derived from the extracellular matrix, a parameter that could be of great relevance to RA pathophysiology.

While the inhibitory effects of CSE on the ROCK2-IRF4 axis might seem surprising in view of previous work demonstrating high total ROCK activity levels in pulmonary arteries and in leukocytes of smokers (Hidaka et al., 2010), the ability of CSE to dampen this axis is consistent with the known capacity of CS to exert a wide-range of immunosuppressive effects. In line with these inhibitory effects, furthermore, nicotine has previously been shown to decrease the frequencies of IL-17 producing cells in a murine model of IBD although IL-22 production in those studies was not evaluated (Liu et al., 2014). Furthermore, the short-term immunosuppressive effects of CS may eventually promote inflammatory sequelae because of associated effects like a decreased ability to fight infections. In this regard, it is important to note that CS exerted greater inhibitory effects on ROCK2 than on ROCK1 activation thus potentially altering the balance between these two isoforms. This finding raises the possibility that the elevated levels of ROCK activity in smokers may reflect primarily increases in ROCK1. The development of tools designed to specifically assess the activity of the two isoforms as well as the generation of genetic approaches aimed at dissecting the precise role of the two isoforms will be critical to shed insights into this possibility.

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