

**Healthy, but not RSV-infected, lung epithelial cells profoundly inhibit T-cell activation**

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## **ABSTRACT**

**Rationale:** Respiratory viruses, including respiratory syncytial virus (RSV), can cause asthma exacerbations and bronchiolitis. Both conditions are associated with enhanced cognate immune responses and inflammation and reduced immune regulation. Lung epithelial cells (LECs) can contribute to antiviral and allergic immune responses while gut epithelial cells can inhibit effector T-cell responses.

**Objectives:** Here, we asked whether healthy LECs regulate antigen-specific T-cell responses and if this regulation is lost during RSV-infection.

**Methods:** LA4-cells, a murine LEC line, in some experiments infected with RSV, or primary murine LECs, were co-cultured with ovalbumin-specific, T-cell-receptor-transgenic CD4<sup>+</sup> T-cells from DO11.10 mice and ovalbumin-pulsed, bone marrow-derived dendritic cells (DC) to assess T-cell proliferation by flow cytometry and cytokine production.

**Results:** The presence of LECs abrogated DC-induced T-cell proliferation and significantly reduced T-cell cytokine release. These effects of LECs were predominantly contact-dependent, primarily affected T-cells directly and were partly mediated by TGF- $\beta$ . Soluble factors and DC-mediated effects also contributed to T-cell inhibition. RSV-infection of LECs reduced their inhibitory capacity in an infection dose dependent manner. This was independent of pro-inflammatory cytokines released by infected LECs, but in part due to toll-like receptor activation and to infection-induced cell death.

**Conclusion:** Healthy LEC are potent inhibitors of T-cell activation, however this regulatory function is lost after RSV infection. These findings suggest a central role for LECs in maintaining the tolerogenic environment of healthy lungs. Loss of this regulatory capacity after viral infection may allow development of excessive cognate immune responses and pulmonary inflammation.

## INTRODUCTION

Respiratory viruses, including respiratory syncytial virus (RSV), are the most important triggers of asthma exacerbations. [1, 2] In infants respiratory viruses can cause severe bronchiolitis, [3] which is associated with an increased risk of asthma development in childhood. [4, 5] Asthma exacerbations and bronchiolitis are thought to be due, at least in part, to reduced immune regulation in the normally tolerogenic environment of the lung and subsequent failure to maintain tolerance to environmental antigens, resulting in excessive and aberrant T-cell responses. [6]

The mucosa of the lower respiratory tract (LRT), which mainly consists of epithelial cells, provides a physical and functional barrier against inhaled pathogens, allergens and particulates. In respiratory viral infections, this barrier is breached and lung epithelial cells (LECs) are the main port of entry for viruses and their main site of replication. LECs are in close contact with a variety of immune cells including antigen-presenting cells such as dendritic cells (DCs), and intraepithelial lymphocytes.[6] Recently, it has been recognised that LECs can contribute to antiviral immune responses. Upon viral infection LECs express Type 1 interferons (IFN), which induce antiviral proteins and LEC apoptosis, activate plasmacytoid DCs and promote cellular antiviral responses,[7, 8] as well as pro-inflammatory cytokines and chemokines. In addition, virus infected LECs express co-stimulatory molecules which may modulate CD8+ T-cell responses.[9] In asthmatic airways, LECs over-express IL-13, a Th-2 cytokine that further enhances allergic inflammation and mucous hyperplasia.[10] In contrast, gut epithelial cells of the colon have been shown to inhibit CD4+ T-cell proliferation. [11] Whether such immune regulatory effects of epithelial cells are unique to the gut, or if they occur in other mucosal sites is not known.

These observations suggest that LECs may be central to both the maintenance of the tolerogenic immune environment of healthy lungs and the switch to inflammation and increased cognate immune responses following respiratory viral infections. We therefore tested the hypothesis that healthy LECs inhibit T-cell activation, and that this inhibition is lost in RSV-infection.

## MATERIAL AND METHODS

Please see online repository for detailed information.

### Mice

Female BALB/c mice aged 8-10 weeks (Charles River Laboratory, Margate, UK) and DO11.10 mice [12] (The Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen free conditions and used as sources of bone marrow derived DCs (BM-DC) and T-cell receptor (TCR) transgenic, ovalbumin (OVA)-specific, CD4+ T-cells (DO11.10-T-cells), respectively, and under experimental protocols approved by the Home Office, London, UK.

### Virus

Plaque-purified, human RSV-A2 (LGC Promochem) and a transgenic RSV strain expressing green fluorescent protein (GFP-RSV),[13] (Dr ME Peeples, Ohio State University) were grown in HEp-2 cells (LGC Promochem).

### **Generation of BM-DC**

Bone marrow cells from femurs were cultured in the presence of recombinant murine GM-CSF (Life Technologies, Paisley, UK) for 10-12 days, when resultant BM-DC were harvested. [14]

### **Lung epithelial cells**

LA4-cells, a murine, lung, alveolar type-II epithelial cell line [15] (LGC Promochem) were grown to confluence in Ham's F-12 medium (Invitrogen).

Primary LECs were isolated from lungs of naïve BALB/c mice by dispase II digestion and subsequent depletion of contaminating mononuclear cells using anti-CD45, anti-CD32/16, anti-CD31 and anti-CD90 antibody and MACS beads (Miltenyi).[16] Isolated primary LECs were cultured in complete RPMI 1640 medium for three days before use in co-culture experiments.

To assess the ability of LECs to inhibit DC-induced T-cell proliferation, LA4-cells or primary LECs ( $2 \times 10^5$  /well) were cultured alone for 24 hours, when DC/T-cell co-cultures were added directly to LECs. In some co-cultures, direct contact of LA4-cells with DCs or T-cells was prevented by Transwell chambers (Costar, Fisher Scientific Ltd). In antibody neutralization assays, LA4-cells were incubated prior to and during co-culture with DC/T-cells with anti-mouse PD-1 antibody (Clone J43, eBioscience), anti-mouse TGF- $\beta$  (1,2,3) (Clone 1D11, R&D Systems) or appropriate isotype controls.

In infection experiments, LA4-cells were inoculated with RSV, UV-inactivated RSV (UV-RSV) or they remained uninfected. After 24 hours, LA4-cells were washed and DC/T-cells were added. To dissect RSV-effects on the inhibitory capacity of LECs, LA4-cells were pre-treated with TSLP, GM-CSF or IL-6 (Invitrogen) or toll-like receptor (TLR) agonists: lipo-polysacchride (LPS) from E coli serotype 055 :B5, Poly I:C (both from Sigma-Aldrich, Dorset, UK), CpG-ODN1826 (5'-TCCATGACGTTCTGACGTT-3') or control ODN1982 (Life Technologies).

### **T-cell proliferation assays**

Following depletion of CD11c+ cells, splenic CD4+ T-cells were isolated from DO11.10 mice, both by MACS beads (Miltenyi). Purified DO11.10-T-cells ( $5 \times 10^5$ /well) were stained with carboxy fluorescein succinimidyl ester (CFSE) (Life Technologies), co-cultured with BM-DC ( $1 \times 10^5$ /well) pulsed with OVA or PBS, and layered onto LECs or medium. After four days DC/T-cells were harvested and reduction in CFSE fluorescence, indicating T-cell proliferation, was determined by flow cytometry. [17] In some experiments, proliferation of T-cells from BALB/c mice was induced by anti-CD3 antibody and irradiated BM-DC.

### **Flow cytometry**

Following FC-receptor blockade cells were stained with antibodies to mouse DO 11.10-TCR, CD62L (Caltag); Foxp3, GITR, PDL-1 and PDL-2 (all eBioscience); CD4, CD25, CD3 or isotype controls (all BD Biosciences). Samples were acquired using a LSR flow cytometer and CellQuest software (both BD Biosciences) and analysed using WinList software (Verity Software).

### **ELISA**

Cytokine concentrations in culture supernatants were assessed using the following ELISA kits: mouse GM-CSF, TSLP and TGF- $\beta$  (all R&D Systems), mouse IL-6 and TNF- $\alpha$  (both

Biosource); and antibody pairs: mouse IL-1, IL-4, IL-5, IL-10, IL-12p70, IL-17, and IFN- $\gamma$  with protein standards (all BD Biosciences). OD values at 450 nm were measured by MRXII spectrophotometer (Dy nex) with Revelation F3.21-software (ThermoBioAnalysis SA).

### Statistical analysis

Results are expressed as means  $\pm$  SEM. All data were normally distributed (GraphPad InStat) and compared by ANOVA followed by Bonferroni test if p-values were significantly different, using GraphPad Prism 4.02 (GraphPad Software). Differences were considered to be significant at  $p < 0.05$ .

## RESULTS

### LEC inhibit DC-induced T-cell activation

To assess whether LECs have the ability to influence antigen-specific T-cell activation, we analyzed CFSE-labelled, DO11.10-T-cells that were co-cultured with OVA-pulsed BM-DC. After 4 days of culture, strong T-cell proliferation was detected. DO11.10-T-cells by themselves did not proliferate. If DC and T-cells were co-cultured on confluent layers of LA4-cells, the proliferation of DO11.10-T-cells was abrogated (fig 1a). Parallel results were obtained with primary murine LECs, which increased the percentage of non-proliferated T-cells from  $38.09 \pm 3.0\%$  to  $96.01 \pm 0.91\%$  (see online repository). This inhibition of T-cell proliferation was independent of the DC/T-cell ratio used in co-cultures (data not shown) but did depend on LA4-cell numbers seeded at the beginning of culture (fig 1b).

Assessing T-cell and DC-derived cytokines, we found that DO11.10-T-cells, BM-DC or LA4-cells cultured alone did not secrete cytokines with exception of low GM-CSF levels in LA4-cell cultures (data not shown). In contrast, in DC/T-cell co-cultures the T-cell cytokines IFN- $\gamma$ , IL-5, and IL-17 and TNF- $\alpha$  were produced (fig 1c). The presence of LA4-cells during DC/T-cell co-cultures significantly inhibited production of these cytokines, but increased concentrations of GM-CSF and TSLP, which were low or undetectable in the absence of LA4-cells. IL-4, IL-10, and IL-12 were not detected in any of the cultures.

### Direct cell contact and soluble mediators both contribute to T-cell inhibition by LECs.

To determine whether the inhibition of T-cell proliferation by LECs is cell contact dependent or mediated by soluble factors, we compared T-cell proliferation after co-culture with LA4-cells which were either in direct contact with DC/T-cells or separated from these in transwell chambers. Direct cell contact of DC/T-cell co-culture with LA4-cells completely prevented T-cell proliferation, while co-culture with LA4-cells without direct contact but in the same medium reduced T-cell proliferation by about 30% (fig 2a).

Programmed death (PD)-ligand (PD-L)1 and PD-L2 which bind to PD-1 on T-cells and TGF- $\beta$ , are all expressed by epithelial cells,[9, 18] as confirmed here (data not shown). These molecules have all been implicated in the inhibition of effector T-cell responses. [19, 20] To determine their involvement in LEC-induced T-cell inhibition, we used blocking antibodies to PD-1 and TGF- $\beta$  in LA4-cell/T-cell/DC co-cultures (fig. 2b). These antibodies did not affect T-cell proliferation in control cultures. In the presence of LA4-cells, PD-1 blocking had no effect on

LEC mediated T-cell inhibition. In contrast, addition of anti-TGF- $\beta$  antibody, but not its isotype control (not shown), restored some degree of T-cell proliferation.

### **LECs primarily inhibit T-cells directly and to a lesser extent via DCs.**

The inhibition of DC-induced T-cell proliferation by LECs could be a direct effect on T-cells or mediated by DCs. To resolve this question, BM-DCs and DO11.10-T-cells were separately cultured with LA4-cells for 24 hours or exposed to control medium. After removal of LA4-cells, DCs and T-cells were co-cultured and T-cell proliferation was assessed 3 days later (fig 3a). LEC pre-treatment of both DCs and T-cells or of T-cells alone markedly inhibited T-cell proliferation, whereas pre-treatment of DCs only with LECs only inhibited T-cell proliferation to a small degree. This suggests that T-cell inhibition by LECs is primarily due to direct effects on T-cells. To confirm this hypothesis we induced T-cell proliferation with anti-CD3 antibody, giving the TCR signal required for activation, and irradiated DCs, that provided co-stimulatory signals but were unable to respond to LA4-cells (fig 3b). If LA4-cells were added the otherwise robust T-cell proliferation was prevented almost completely, demonstrating that LECs can directly inhibit T-cell activation. This was confirmed using primary LECs (see online repository).

### **LECs induce regulatory T-cells (Tregs) in DC/T-cell co-cultures.**

Next, investigated we possible mechanisms for the lack of T-cell proliferation in the presence of LECs. In addition to T-cell anergy, induction of Tregs by LECs could inhibit T-cell proliferation. We therefore examined the expression of Foxp3, a marker of Tregs, in T-cells co-cultured with DCs in the presence or absence of LA4-cells (fig 4a, b). Foxp3 was expressed both in CD25+ and CD25- CD4+ T-cells (data not shown) and the level of expression in naïve T-cells did not change after stimulation with OVA-pulsed BM-DC. When LA4-cells were added directly to these cultures, the percentage of Foxp3+ DO11.10-T-cells trebled. In the absence of direct contact of DC/T-cells with LECs in transwell cultures the induction of Foxp3+ DO11.10 T-cells was less pronounced (from  $4.47 \pm 0.2\%$  to  $8.06 \pm 0.5\%$ ,  $p < 0.05$ ,  $n = 6$ ). Further, expression of glucocorticoid-induced tumor necrosis factor receptor (GITR), another marker of Tregs, was increased in T-cells after exposure to LA4-cells (see online repository). These findings indicate that LECs induced cells with a Treg phenotype. To determine whether LEC-exposed T-cells, which contain the Foxp3+ population, have immune suppressive activity, we assessed their influence on T-cell proliferation in secondary DC/T-cell co-cultures (fig 4c). Here, DO11.10-T-cells were stimulated to robust proliferation by BM-DC and this remained unchanged after addition of control T-cells from DC/T-cell cultures without LECs. In contrast, addition of T-cells exposed to LA4-cells significantly inhibited T-cell proliferation in secondary cultures, indicating the presence of functional Tregs. In keeping with the inhibitory function of Tregs, CD62L was up-regulated in DC/T-cell co-cultures in the presence of LECs (see online repository), indicating suppression of T-cell activation.

### **RSV-infection of LA4-cells reduces their T-cell inhibitory capacity.**

LECs are the primary target for RSV-infection in the LRT, which leads to enhanced T-cell responses and inflammation. We therefore asked if RSV-infection of LECs interferes with their ability to inhibit T-cell activation and proliferation. Having ascertained that LA4-cells can be infected with RSV, using GFP-RSV (see online repository), we infected sub-confluent LA4-cells with increasing doses of RSV (MOI of 0.1, 1 and 10), added DC/T-cell co-cultures to these 24 hours later and assessed T-cell proliferation after another 72 hours (fig 5). Inhibition of T-cell proliferation was almost complete in the presence of LA4-cells sham infected with UV-RSV,

whereas infection with live RSV decreased LEC induced inhibition of T-cell proliferation in an infection dose dependent manner. After infection of LA4-cells with RSV at an MOI of 0.1 and 1 T-cell proliferation was partially restored and at an MOI of 10 T-cell inhibition was lost completely.

### **TLR-3/-4 activation and RSV-induced cell death, but not pro-inflammatory cytokines, reduce the T-cell inhibitory capacity of LECs.**

RSV-infection in LECs is known to activate TLR-3 through double stranded RNA and TLR-4 through RSV-F protein, to induce secretion of pro-inflammatory cytokines and to trigger apoptosis and cell death. To determine whether these factors reduce the ability of LECs to inhibit T-cell activation, we initially monitored secretion of pro-inflammatory cytokines by RSV-infected LECs and demonstrate that they have no effect on T-cell inhibition by LECs (see online repository).

In separate experiments, we exposed LA4-cells for 24 hours to the TLR-3 ligand poly I:C, the TLR-4 ligand LPS, or to PBS as a control (fig 6a). CpG 1826, a ligand of TLR-9, which is not activated by RSV, was used to assess if non-viral TLR-activation of LECs also affects their T-cell inhibitory capacity. LA4-cells pre-treated in this way were then used in co-cultures with DC/T-cells and T-cell proliferation was assessed after three days. While CpG 1826 did not have any effect on the ability of LA4-cells to inhibit T-cell proliferation, exposure to either LPS or poly I:C significantly reduced this ability and restored some degree of T-cell proliferation.

Finally, we investigated if the death of LA4-cells contributes to the reduction in T-cell inhibition after RSV-infection. In co-cultures with DCs and T-cells, LA4-cells formed confluent layers, which remained intact up to 48 hours after infection and still showed more than 50% confluence at 72 hours. To assess cell death by trypan blue exclusion assay over the culture period, LA4-cells were cultured in control medium or in conditioned medium from DC/T-cell co-cultures and infected with RSV (MOI = 1) (fig 6b). Without RSV infection, irrespective of the medium used, the percentage of dead cells in LA-4 cultures remained low (<10%) over four days. Following RSV-infection, LA4-cells cultured in control medium had significantly lower rates of cell death than those cultured with conditioned medium from DC/T-cell co-cultures, where the rate of cell death reached  $81.29 \pm 1.61\%$  by day 4. This indicates that LA4-cells are more susceptible to RSV-induced cell death in the presence of mediators secreted by DCs or T-cells. The substantial rate of cell death in LECs after infection probably contributed significantly to the reduction in T-cell inhibition.

## **DISCUSSION**

We aimed to determine whether LECs can inhibit T-cell activation and whether RSV-infection prevents such an inhibition.

To model antigen-specific T-cell responses, naïve DO11.10-T-cells were stimulated with OVA-pulsed BM-DC, resulting in robust T-cell proliferation.[17] Murine, alveolar, type-II epithelial LA4-cells [15] and primary murine LECs were used to represent LRT-epithelial cells. Co-culture of DC/T-cells with confluent layers of LA4-cells or primary LECs abrogated T-cell proliferation and significantly reduced T-cell cytokine production, indicating that these LECs provided inhibitory signals that prevented normal T-cell activation. Such triple co-cultures may well

represent healthy LRT-mucosa, which contains T-lymphocytes and DCs and provides a tolerogenic environment, minimizing inappropriate immune response to harmless inhaled antigens such as allergens.[6]

Delineating the requirements for T-cell inhibition by LECs, we found that LECs had to be present early in co-cultures prior to T-cell activation, that the extent of T-cell inhibition increased with the duration of LEC-exposure and that T-cell inhibition was not fully reversible after removal of LECs (see online repository). Transwell cultures preventing direct cell contact of DC/T-cells with LECs but not exposure to soluble mediators, revealed that direct contact induced almost complete T-cell inhibition, while soluble factors only reduced T-cell proliferation by 30%. This suggests either that LECs express an inhibitory factor on their surface which is also shed into the medium, or that a combination of independent membrane-bound and secreted factors cause T-cell inhibition.

Searching for inhibitory factors, we blocked PD-1 and TGF- $\beta$  by antibody in triple co-cultures. LECs can express co-stimulatory molecules including PDL-1 and PDL-2.[9] These bind to PD-1 on T-cells, a receptor that provides inhibitory signals and that is implicated in Treg-development and T-cell tolerance.[21] Anti-PD-1 treatment did not reduce LEC-induced T-cell inhibition, indicating that PDL-1 and -2 are not involved in the process.

TGF- $\beta$  is secreted by LECs [18] and is a factor in the induction of some Treg-subsets (Th-3 cells). [22] Anti-TGF- $\beta$  treatment resulted in partial restitution of T-cell proliferation, indicating that TGF- $\beta$  plays a role in T-cell inhibition by LECs. Although TGF- $\beta$  can be secreted and act as a soluble factor, on macrophages and natural Tregs it is primarily active locally on the cell surface [20, 23]. This could explain the strong cell contact-dependent inhibition and the weaker effect of soluble mediators, observed here. The limited effect of anti-TGF- $\beta$  treatment on T-cell inhibition may well be due to an inability of the antibody used to neutralise TGF- $\beta$  secreted locally into areas of cell-cell contact.

To determine if the effects of LECs were directly on T-cells or if they were mediated by DCs, T-cells or DC were pre-cultured with LECs prior to DC/T-cell co-cultures. In separate experiments T-cells were stimulated with anti-CD3 antibody and irradiated BM-DCs unable to express inhibitory factors *de novo* upon LEC-contact. Both approaches demonstrated that inhibitory effects of LECs were primarily direct effects on T-cells. However, the pre-culture experiments also demonstrated DC-mediated inhibition of T-cell activation by LECs. Inhibitory effects of LECs on DCs may explain why DCs from healthy lungs are poor inducers of effector T-cell activation [24, 25].

LEC induced T-cell inhibition could be due to clonal deletion, T-cell anergy, and the induction of Tregs. Clonal deletion is unlikely to play a major role in our model since numbers of DO11.10-T-cells, the majority of which belong to the same transgenic, OVA-specific clone, did not decline in the presence of LECs (data not shown). Regulation of T-cell responses in many settings is thought to be due to Tregs. Different subsets of Tregs have been described, including naturally occurring Tregs and inducible Tr1- and Th-3 cells which mediate immune regulation by IL-10 and TGF- $\beta$ , respectively.[26] Expression of Foxp3, the critical transcription factor driving Treg-development, is a hallmark of naturally occurring Tregs and has also been demonstrated in

induced Tregs.[27] Here, 4-5% of naïve or activated T-cells expressed Foxp3 and in the presence of LECs the percentage of Foxp3+ T-cells trebled, indicating the induction of T-cells with a regulatory phenotype. Addition of LEC-exposed T-cells from triple co-cultures to secondary DC/T-cell co-cultures significantly inhibited T-cell proliferation, demonstrating that LA-4-cell exposed T-cells did indeed contain functional Tregs. The induction of Tregs by LECs and the finding that LEC-induced T-cell inhibition is not completely lost even if LEC-contact is ended, suggests that in the healthy LRT, Tregs are generated which may retain their immune suppressive effects even if they migrate out of the mucosa to the regional lymph nodes. Here, they are likely to contribute to the maintenance of normal immune tolerance to environmental antigens.

Since respiratory viruses are major triggers of airway inflammation and asthma exacerbations, we asked if viral infection of LEC reduces their immune regulatory function. RSV-infection of LA4-cells 24 hours prior to addition of DC/T-cell co-cultures reduced T-cell inhibition in a dose-dependent fashion. This effect of RSV-infection cannot be explained by accidental infection of DCs, which would have displayed a reduced ability to induce T-cell proliferation. [28]

If infection was mimicked by treatment of non-infected LA-4-cells with poly I:C (an artificial double-stranded RNA and TLR3 agonist as it occurs during RSV replication) or LPS (a TLR4 agonist like RSV F-protein [29]), T-cell inhibition was significantly reduced in subsequent DC/T-cell co-cultures. This indicates that some TLR-induced pathways in LECs reduce their ability to inhibit T-cell proliferation. In contrast, pro-inflammatory cytokines from RSV-infected LECs do not change their T-cell inhibitory capacity (see online repository).

Considering that LECs can undergo apoptosis and necrosis during viral infection, we asked if RSV-induced death of LEC accounts for the reduction in T-cell inhibitory capacity. Trypan blue exclusion assays revealed that about 50% of LA4-cells died within four days of RSV-infection and that DC/T-cell-derived mediators rendered them even more susceptible to RSV-induced death, suggesting that the latter contributed significantly to the reduced T-cell inhibitory capacity of LECs. This may also apply during natural RSV-infection which induces extensive destruction of LRT-epithelium.[3] In affected areas, normal inhibition of T-cells and DCs may be lost, resulting in excessive, local T-cell responses and subsequent inflammation. This notion is in keeping with the enhanced T-cell stimulatory capacity of pulmonary DCs, observed following RSV-infection,[24] and other respiratory viral infections.[25, 30]

Taken together, our findings suggest that LECs inhibit T-cell activation in healthy airway mucosa and that they induce Tregs which suppress unwanted adaptive immune responses not only in the mucosa but also in the associated regional lymph nodes. Upon respiratory viral infection, e.g. with RSV, the inhibitory capacity of LECs is compromised, allowing local activation of T-cell responses in the respiratory mucosa and consequently airway inflammation. It needs to be recognised that these hypotheses are based on in-vitro studies with murine cells. Thus, further studies in human LEC lines and primary LECs from patients with and without viral infection of the LRT will be required to validate our findings in the clinical setting.

In conclusion, we report that healthy LECs are potent inhibitors of T-cell activation and proliferation and associated cytokine secretion. Our data demonstrate that LECs provide both membrane-bound and soluble inhibitory factors with direct effects on T-cells; suggest that TGF- $\beta$  contributes to this inhibition; and, show that T-cell inhibition is, at least in part, achieved through

the induction of Tregs. After RSV-infection the T-cell inhibitory capacity of LECs is reduced or lost depending on severity of infection. This is partly due to TLR activation in LECs and to cell death.

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None

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#### **FIGURE LEGENDS**

##### **Figure 1: Inhibition of DC-induced, antigen-specific T-cell proliferation and cytokine production by LECs**

(A) CFSE-stained DO11.10 T-cells were cultured alone or co-cultured with OVA-pulsed BM-DC (ratio 5:1) in the presence or absence of LA4-cells ( $5 \times 10^5$  cells per well). DC-induced T-cell proliferation was assessed by flow cytometry measuring CFSE dilution, shown here in dot plots. The percentages indicate non-proliferated T-cells. (B) DC/T-cells were co-cultured with increasing numbers of LA4-cells (+: 1x, ++: 2x, +++: 4x, ++++:  $6 \times 10^5$  cells per well) and T-cell proliferation was assessed and shown as percentages of non-proliferated T-cells. (C) Cytokine concentrations in supernatants of these co-cultures were quantified by ELISA. The graphs show means  $\pm$  SEM from a representative experiment (6 samples per group) of three independent experiments. Significant differences as indicated by horizontal bars: \*\* $p < 0.01$ .

##### **Figure 2: Direct cell contact and soluble mediators both contribute to T-cell inhibition by LEC.**

(A) DC/T-cell co-cultures were performed alone, in direct contact with LA4-cells or in transwell plates separating LA4-cells from DCs and T-cells. (B) Blocking antibodies to PD-1 and TGF- $\beta$  were added at different concentrations to co-cultures of DCs, T-cells and LA4-cells. In both experiments, T-cell proliferation was assessed after 4 days. The graphs depict means  $\pm$  SEM of percentages of non-proliferated cells from a representative experiment (6 samples per group) of three independent experiments. Significant differences: \* $p < 0.05$ , \*\* $p < 0.01$ .

**Figure 3: LECs inhibit T-cells directly and to a lesser extent via DCs.**

(A) OVA-pulsed BM-DC and CFSE-labelled DO11.10 T-cells were cultured separately with LA4-cells (LA4 DC, LA4 T-cells) or control medium (Normal DC, Normal T-cells) for 24 hours. Subsequently, these DC and T-cells were co-cultured in different combinations. (B) Irradiated BM-DC plus anti-murine-CD3 antibody were used to induce proliferation of CFSE-labelled, splenic CD4 + T-cells from naïve BALB/c mice in the presence or absence of LA4-cells. In both experiments T-cell proliferation was measured after 72 hours of DC/T-cell co-culture. The graphs depict means  $\pm$  SEM of percentages of non-proliferated cells from a representative experiment (6 samples per group) of three independent experiments. Significant differences as indicated by horizontal bars: \* $p < 0.05$ , \*\* $p < 0.01$ .

**Figure 4. Induction of Tregs in DC/T-cells co-cultures exposed to LECs.**

Naïve DO11.10 T-cells were co-cultured with OVA-pulsed BM-DC in the presence or absence of LA4-cells. After 48 hours of culture Foxp3 expression in DO11.10 T-cells was assessed by intracellular staining and flow cytometry. (A) Dot plots show Foxp3 expression in DO11.10 T-cells; (B) The graph shows means  $\pm$  SEM of percentages of Foxp3+ cells of total CD4+ T-cells (after subtraction of isotype controls) from a representative experiment of three independent experiments. Significant differences as indicated by horizontal brackets: \*\* $p < 0.01$ . (C) To determine if LA4 cell primed T-cells inhibit T-cell proliferation we transferred T-cells from DC/T-cell co-cultures with (LA4 exposed) or without LA4-cells to secondary DC/T-cell co-cultures and measured T-cell proliferation in these secondary cultures after 4 days. The graph depicts means  $\pm$  SEM of percentages of non-proliferated cells from a representative experiment (6 samples per group) of three independent experiments. Significant differences as indicated by horizontal brackets: \*\* $p < 0.01$ .

**Figure 5. RSV infection of LA4-cells reduces their T-cell inhibitory capacity**

24 hours prior to co-culture with DC/T-cells, LA4 cell were infected with increasing doses of RSV (MOI = 0.1-10) or with UV-inactivated RSV as a control. T-cell proliferation was determined by CFSE assay after 4 days of culture. The graph depicts means  $\pm$  SEM of percentages of non-proliferated cells from a representative experiment (6 samples per group) of three independent experiments. Significant differences as indicated by horizontal brackets: \*\* $p < 0.01$ .

**Figure 6: Influence of TLR ligands and cell death on LEC-induced T-cell inhibition**

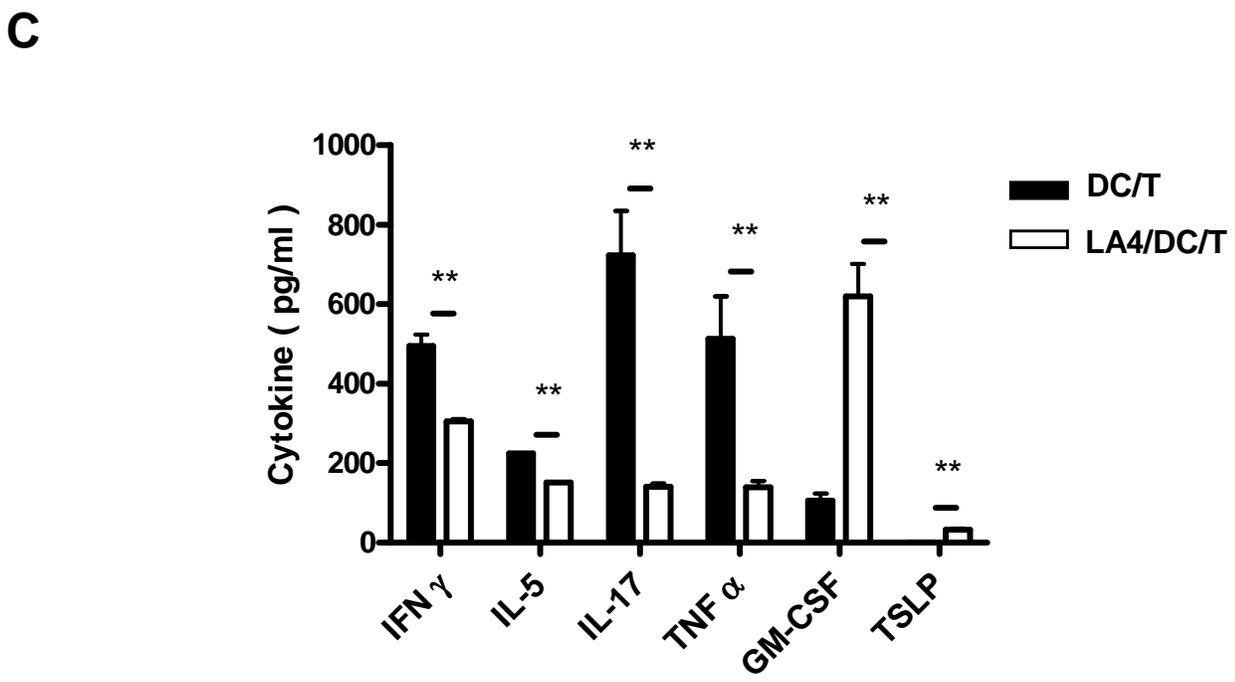
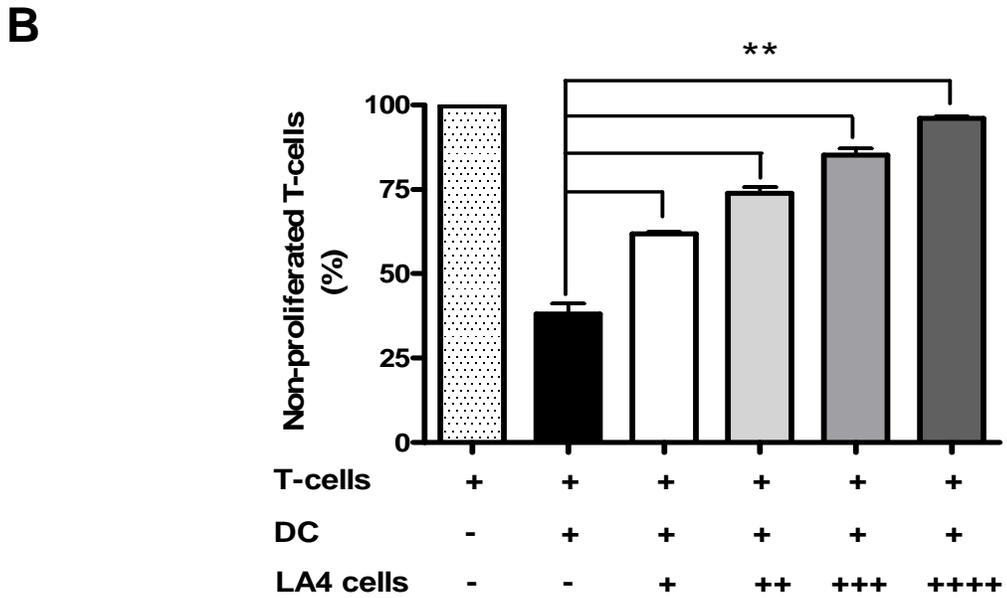
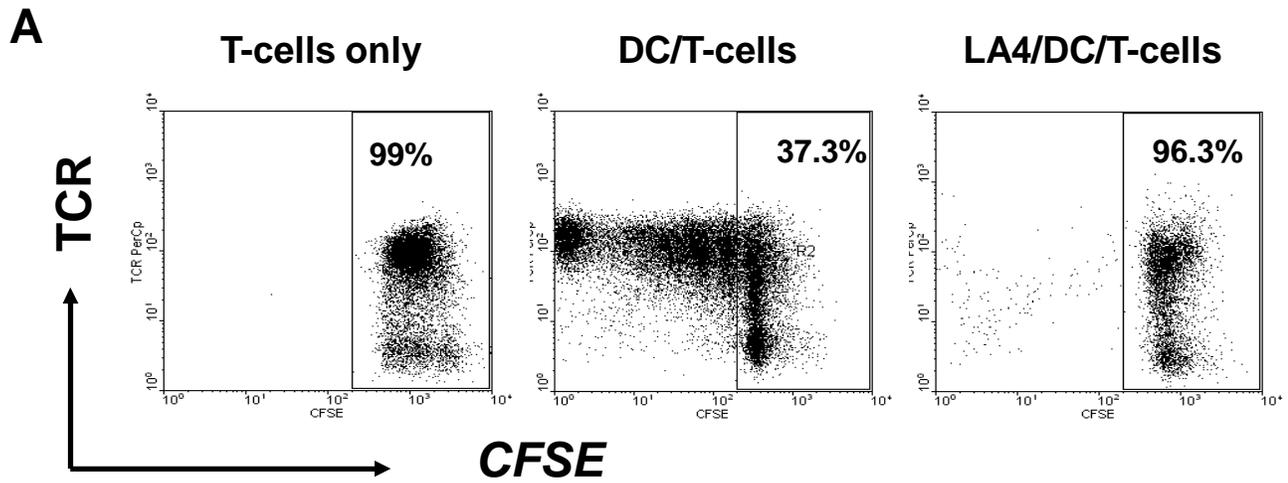
(A) LA4-cells were pre-incubated for 24 hours with the following TLR ligands: LPS, poly I:C and CPG 1826 (all at 10  $\mu\text{g/ml}$ ) or PBS as a control, washed and co-cultured with DC/T-cells. T-cell proliferation was assessed after 72 hours by CFSE assay. (B) LA4-cells cultured in control medium or conditioned medium from DC/T-cell co-cultures were infected with RSV (MOI = 1) and cell viability was monitored over 4 days by trypan blue exclusion. The graphs depict means  $\pm$  SEM of percentages of non-proliferated T-cells (A) or percentage of dead cells (B) from a representative experiment (6 samples per group) of three independent experiments. Significant differences as indicated by horizontal bars (A) or vertical brackets (B): \* $p < 0.05$ , \*\* $p < 0.01$ .

## REFERENCES

- 1 Johnston SL, Pattermore PK, Sanderson G, *et al.* Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995;310(6989):1225-9.
- 2 Wark PA, Johnston SL, Moric I, *et al.* Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. *Eur Respir J* 2002;19(1):68-75.
- 3 Smyth RL and Openshaw PJ. Bronchiolitis. *Lancet* 2006;368(9532):312-22.
- 4 Sigurs N, Gustafsson PM, Bjarnason R, *et al.* Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am J Respir Crit Care Med* 2005;171(2):137-41.
- 5 Stein RT, Sherrill D, Morgan WJ, *et al.* Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 1999;354:541-545.
- 6 Lambrecht BN and Hammad H. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat Rev Immunol* 2003;3(12):994-1003.
- 7 Stetson DB and Medzhitov R. Type I interferons in host defense. *Immunity* 2006;25(3):373-81.
- 8 Takaoka A, Hayakawa S, Yanai H, *et al.* Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 2003;424(6948):516-23.
- 9 Stanciu LA, Bellettato CM, Laza-Stanca V, *et al.* Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines. *J Infect Dis* 2006;193(3):404-12.
- 10 Temann UA, Laouar Y, Eynon EE, *et al.* IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. *Int Immunol* 2007;19(1):1-10.
- 11 Cruickshank SM, McVay LD, Baumgart DC, *et al.* Colonic epithelial cell mediated suppression of CD4 T cell activation. *Gut* 2004;53(5):678-84.
- 12 Robertson JM, Jensen PE and Evavold BD. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J Immunol* 2000;164(9):4706-12.
- 13 Hallak LK, Spillmann D, Collins PL, *et al.* Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. *J Virol* 2000;74(22):10508-13.
- 14 Garrigan K, Moroni-Rawson P, McMurray C, *et al.* Functional comparison of spleen dendritic cells and dendritic cells cultured in vitro from bone marrow precursors. *Blood* 1996;88(9):3508-12.
- 15 Stoner GD, Kikkawa Y, Kniazeff AJ, *et al.* Clonal isolation of epithelial cells from mouse lung adenoma. *Cancer Res* 1975;35(8):2177-85.
- 16 Corti M, Brody AR and Harrison JH. Isolation and primary culture of murine alveolar type II cells. *Am J Respir Cell Mol Biol* 1996;14(4):309-15.
- 17 Wang H, Peters N, Laza-Stanca V, *et al.* Local CD11c+ MHC class II- precursors generate lung dendritic cells during respiratory viral infection, but are depleted in the process. *J Immunol* 2006;177(4):2536-42.
- 18 Alcorn JF, Rinaldi LM, Jaffe EF, *et al.* Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic airway disease. *Am J Respir Crit Care Med* 2007;176(10):974-82.
- 19 Latchman Y, Wood CR, Chernova T, *et al.* PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2(3):261-8.
- 20 Nakamura K, Kitani A and Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194(5):629-44.
- 21 Keir ME, Francisco LM and Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol* 2007;19(3):309-14.

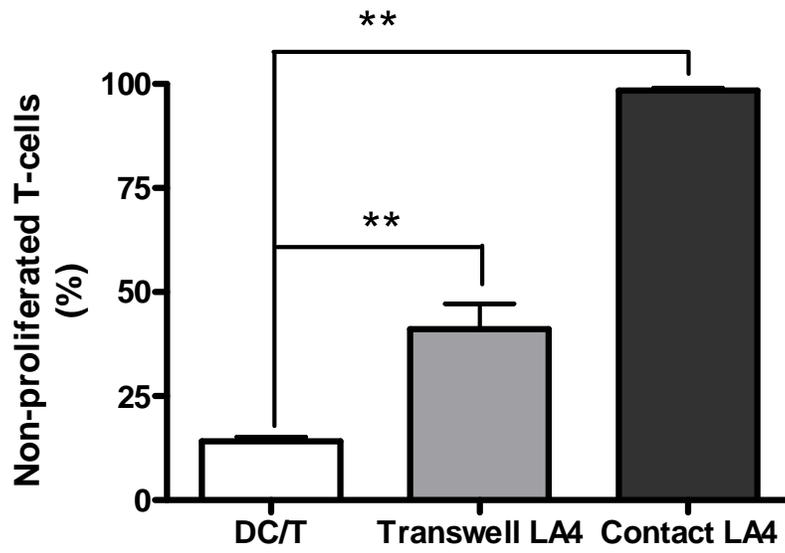
- 22 von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005;6(4):338-44.
- 23 Yehualaeshet T, O'Connor R, Green-Johnson J, *et al.* Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. *Am J Pathol* 1999;155(3):841-51.
- 24 Beyer M, Bartz H, Horner K, *et al.* Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. *J Allergy Clin Immunol* 2004;113(1):127-33.
- 25 Brimnes MK, Bonifaz L, Steinman RM, *et al.* Influenza Virus-induced Dendritic Cell Maturation Is Associated with the Induction of Strong T Cell Immunity to a Coadministered, Normally Nonimmunogenic Protein. *J Exp Med* 2003;198(1):133-44.
- 26 Umetsu DT and DeKruyff RH. The regulation of allergy and asthma. *Immunol Rev* 2006;212:238-55.
- 27 Zheng Y and Rudensky AY. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol* 2007;8(5):457-62.
- 28 Guerrero-Plata A, Casola A, Suarez G, *et al.* Differential response of dendritic cells to human metapneumovirus and respiratory syncytial virus. *Am J Respir Cell Mol Biol* 2006;34(3):320-9.
- 29 Kurt-Jones EA, Popova L, Kwinn L, *et al.* Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000;1(5):398-401.
- 30 Grayson MH, Ramos MS, Rohlfing MM, *et al.* Controls for lung dendritic cell maturation and migration during respiratory viral infection. *J Immunol* 2007;179(3):1438-48.

**Figure 1**

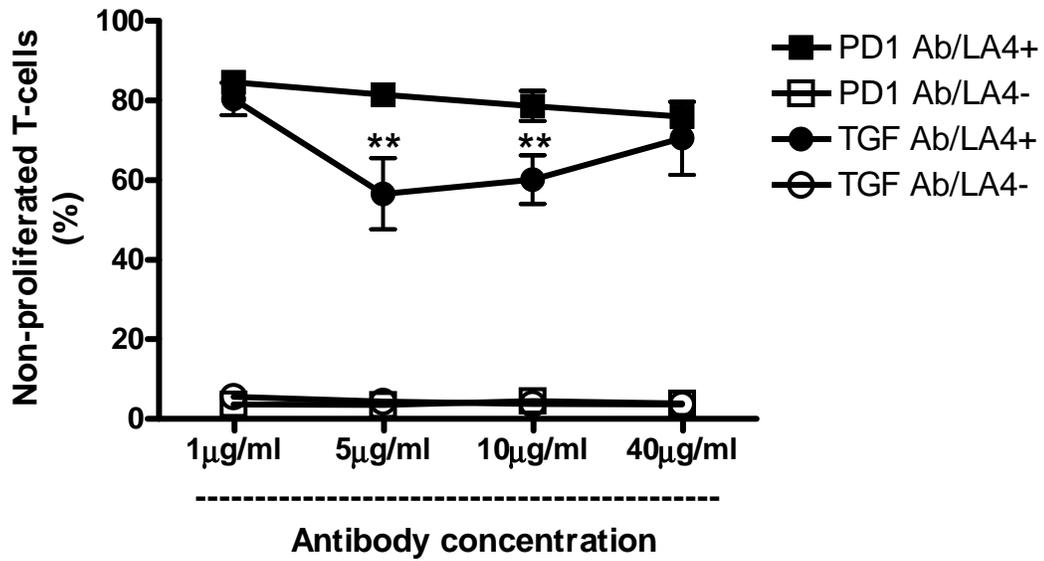


**Figure 2**

**A**

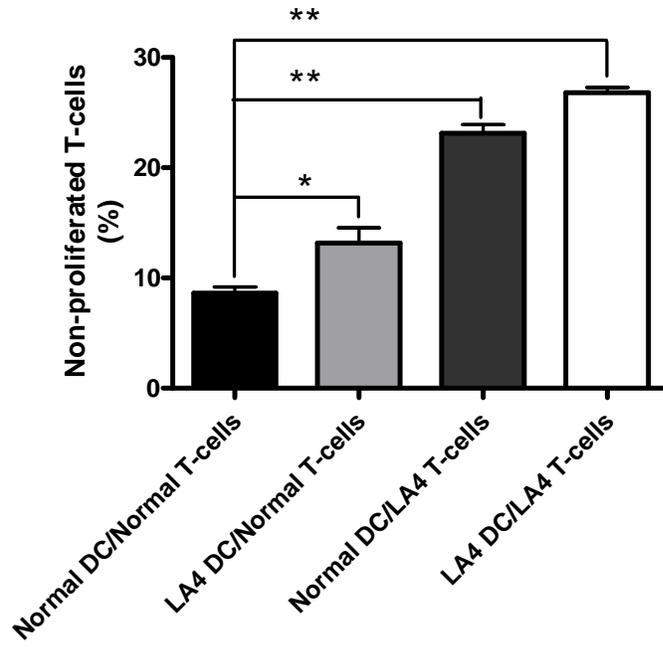


**B**

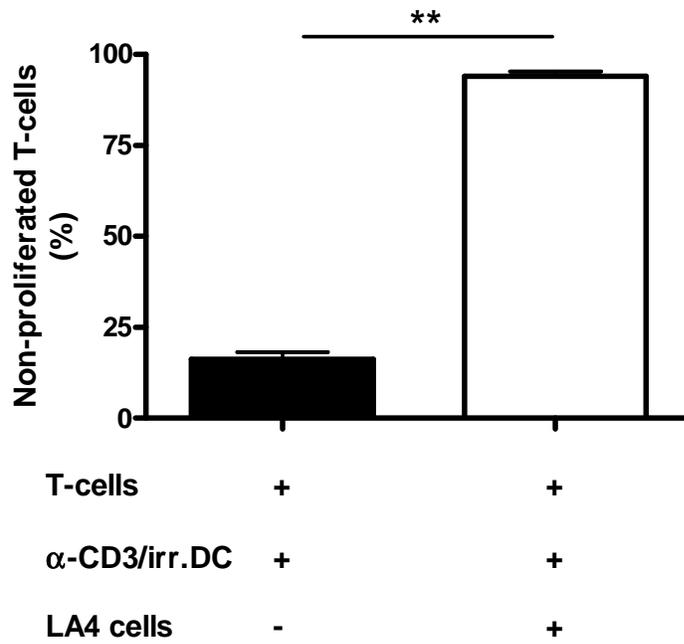


**Figure 3**

**A**

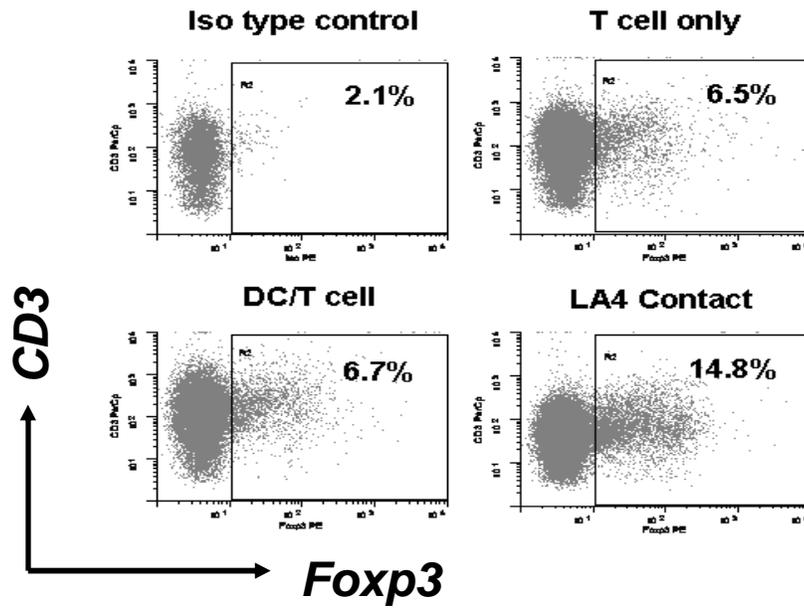


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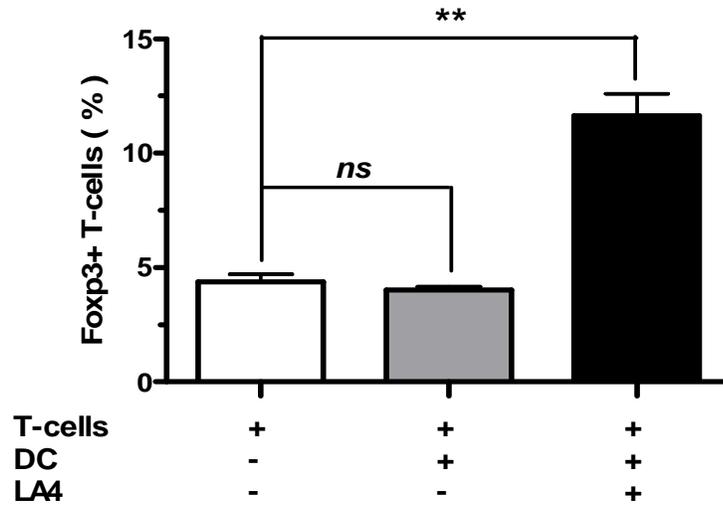


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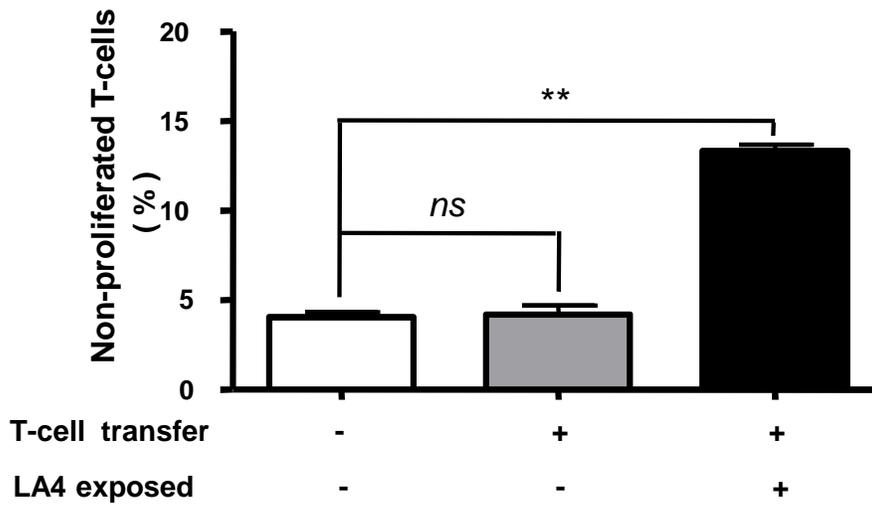
**A**



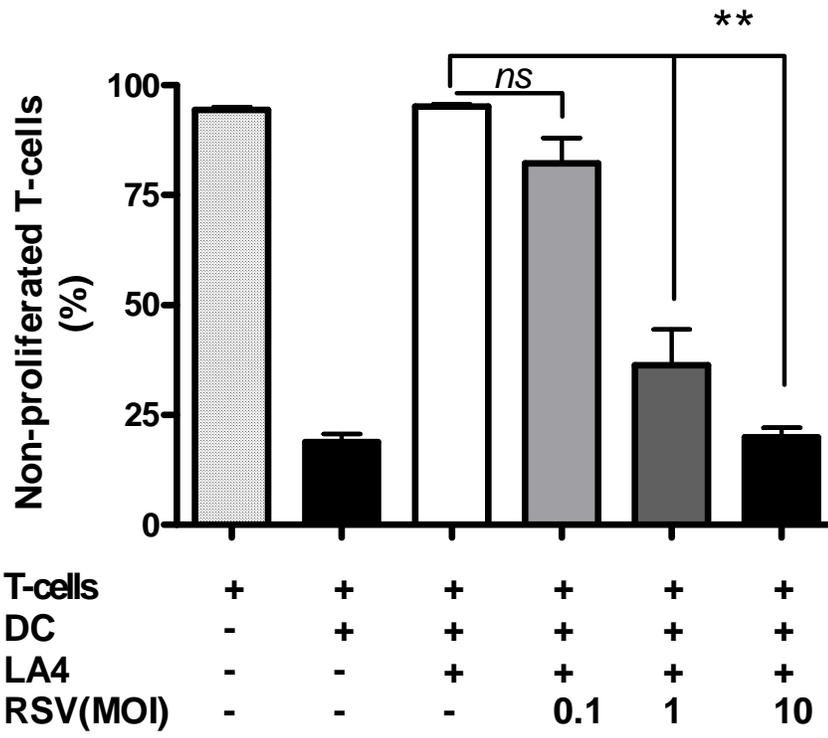
**B**



**C**

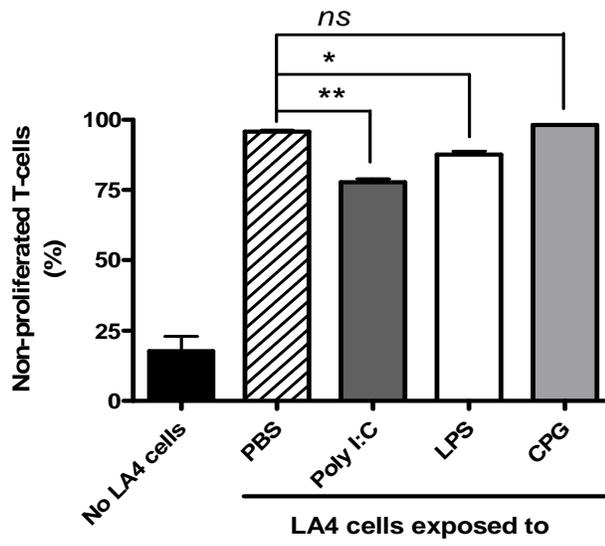


**Figure 5**



**Figure 6**

**A**



**B**

