

# Alveolar Epithelial Type II Cells Induce T Cell Tolerance to Specific Antigen<sup>1</sup>

Bernice Lo,\* Soren Hansen,<sup>†</sup> Kathy Evans,\* John K. Heath,<sup>‡</sup> and Jo Rae Wright<sup>2\*</sup>

The lungs face the immunologic challenge of rapidly eliminating inhaled pathogens while maintaining tolerance to innocuous Ags. A break in this immune homeostasis may result in pulmonary inflammatory diseases, such as allergies or asthma. The observation that alveolar epithelial type II cells (Type II) constitutively express the class II MHC led us to hypothesize that Type II cells play a role in the adaptive immune response. Because Type II cells do not express detectable levels of the costimulatory molecules CD80 and CD86, we propose that Type II cells suppress activation of naive T cells. Purified murine Type II cells were unable to activate T cells to specific Ag or in an alloreactive assay. Although IFN- $\gamma$  treatment up-regulated class II MHC expression, it did not alter the ability of the Type II cells to activate T cells. Rather, the Type II cells were able to suppress T cells from subsequent activation to specific Ag in an Ag-dependent manner. Priming T cells with Type II cells and Ag resulted in T cells that were suppressed to further activation, even after removal from the Type II cells. Thus, Type II cells of the lung help tolerize T cells to nonpathogenic environmental Ags. *The Journal of Immunology*, 2008, 180: 881–888.

**D**uring breathing, the lung is exposed to a constant barrage of pathogens and particles that must be rapidly eliminated by the pulmonary immune system without generating an over-exuberant inflammatory response that could damage the delicate alveolar epithelium and impair gas exchange. In the normal lung, three major cell types comprise the alveolar architecture: the alveolar macrophage, a mobile immune cell that avidly phagocytoses pathogens and particulates; the squamous alveolar type I cells, across which gas exchange occurs; and the cuboidal alveolar epithelial type II cell (Type II).<sup>3</sup> The Type II cell is most widely known as the source of pulmonary surfactant, a lipid and protein complex that reduces surface tension and plays a role in lung host defense (1). In addition to its functions in surfactant homeostasis, Type II cells are also capable of alveolar fluid balance regulation, proliferation, epithelial repair, and differentiation into alveolar epithelial type I cells, modulation of immune cells, and host defense (2).

Because the alveolar epithelial type II cell is found at this interface between the outside air and the pulmonary vasculature, it is in an optimal position for host defense. Type II cells secrete antimicrobial proteins, such as lysozyme, and complement components (e.g., C2, C3, C4, and C5) (3, 4). Additionally, Type II cells have been found to produce a variety of cytokines and chemokines

(2, 5, 6). The fact that Type II cells constitutively express the class II MHC (MHC II) (7, 8) is consistent with the possibility that Type II cells function in the adaptive immune response, as the function of MHC II is Ag presentation to T cells. However, Type II cells have not been found to activate naive T cells or express the necessary costimulatory molecules for T cell activation (9). For optimal T cell activation, costimulation (signal 2) by ligation of CD28 on T cells, in addition to presentation of Ag on MHC II (signal 1), is necessary (10, 11). The ligands for CD28 on the APC are CD80 (B7-1) and CD86 (B7-2). Presentation of Ag on MHC II (signal 1) to T cells in the absence of costimulatory molecules (signal 2) is the hallmark for anergy induction, a form of tolerance (11). Therefore, we hypothesize that Type II cells may be able to induce tolerance in T cells.

In this study, using a murine model, we confirm that Type II cells lack detectable costimulatory molecules and do not effectively activate T cells. We further show that Type II cells suppress subsequent T cell activation to specific Ag, which is indicative of tolerance. Thus, we propose that Type II cells tolerize T cells in the lung, and this mechanism of tolerance by Type II cells may be one of the ways in which the lung suppresses a local inflammatory immune response to some of the innocuous Ags we inhale.

## Materials and Methods

### Animals

DO11.10 TCR transgenic mice and BALB/c mice were obtained from The Jackson Laboratory. C57BL/6 mice were obtained from Charles River Laboratories. SP-C GFP mice (H-2<sup>k</sup> haplotype) (provided by John K. Heath, University of Birmingham, Birmingham, U.K.) were bred and backcrossed into C57BL/6 and BALB/cJ mice at Duke University for at least seven generations. SP-C GFP mice express the GFP transgene under the control of the surfactant protein C (SP-C) promoter. The transgene construct contains the 3.7 kb described previously (12) of the human surfactant protein C promoter region followed by GFP cDNA sequence and the SV40 small T intron and poly(A) (0.4 kb) from pKC4. GFP gene was amplified using the following primers: 5' GGA TGT CGA CTG CAG CCA ATA TG 3' and 5' CTT GAA TTC CTG CAG GTC GA 3'. The amplified fragment was digested with *Eco*R1 and *Sal*I and cloned into the SPC-GFP construct contained in the vector pUC18:Ampr. The SP-C GFP line was generated by pronuclear microinjection of the SPC-GFP construct linearized with *Sac*I. All animal procedures were performed according to local and National Institutes of Health guidelines and were approved by the Duke University Institutional Animal Care and Use Committee.

\*Department of Cell Biology, Duke University Medical Center, Durham, NC 27710;

<sup>†</sup>Department of Immunology and Microbiology, University of Southern Denmark, Odense, Denmark; and <sup>‡</sup>School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom

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<sup>2</sup> Address correspondence and reprint requests to Dr. Jo Rae Wright, Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. E-mail address: j.wright@cellbio.duke.edu

<sup>3</sup> Abbreviations used in this paper: Type II, alveolar epithelial type II cell; MHC II, class II MHC; BMDC, bone marrow-derived dendritic cell; ICOSL, inducible T cell costimulator ligand; PD-L1, programmed death-1 ligand 1; PD-L2, programmed death-1 ligand 2; SP-C, surfactant protein C.

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### Type II cell isolation

Type II cells were isolated from C57BL/6 mice by a procedure modified from Corti et al. (13). For some experiments, mice were i.v. injected once a day for 3 days with IFN- $\gamma$  ( $10^5$  U; 150–175  $\mu$ l) or saline (150–175  $\mu$ l) as a control. On day 4, Type II cells were isolated and used. In brief, the lungs were perfused with a saline/heparin (200 U/ml) solution. Perfused lungs were lavaged once with 1–2 ml of Dispase (BD Biosciences), filled with fresh Dispase via a tracheal catheter, and allowed to collapse naturally. Low melt agarose (1%, 0.4 ml, 42°C) was instilled, and the lungs were immediately covered with crushed ice for 2–3 min. The lungs were removed and incubated in 2 ml of Dispase at room temperature for 45 min. Then the lungs were teased apart and treated with DNase I (Roche). The digest was successively filtered through a 40  $\mu$ m strainer and a 15  $\mu$ m nylon mesh. The cells were panned on mouse IgG (Equatech) coated petri dishes for 1 h at 37°C, and nonadherent cells were collected. Contaminating leukocytes were depleted using biotinylated-anti-CD45, -CD11b, and -CD11c Abs (BD Pharmingen) and streptavidin paramagnetic microbeads (Miltenyi Biotec). Purities were judged by Papnicolaou staining and averaged  $71 \pm 2\%$  (means  $\pm$  SEM) for six independent isolations.

To obtain Type II cells from SP-C GFP mice, lungs were digested and filtered as above. After the filtration step, the cells were FACS sorted for high GFP, high side-scatter cells (Type II cells). Purities were determined by flow cytometry reanalysis and averaged  $89 \pm 1\%$  (means  $\pm$  SEM) for 20 isolations.

### Generation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated as described previously by Inaba et al., and as modified by Brinker et al. (14, 15). In brief, marrow from the tibia, femur, and humerus bones of mice were harvested, cultured in RPMI 1640 supplemented with 5% FCS, antibiotics, and 50  $\mu$ M 2-ME plus 5% GM-CSF conditioned medium for 6 days. Loosely attached cells were harvested and negatively selected with biotinylated-Gr-1 Abs (BD Pharmingen) and streptavidin paramagnetic microbeads (Miltenyi Biotec).

### Specific Ag presentation assay

CD4<sup>+</sup> T cell hybridomas (H-2<sup>b</sup> haplotype) specific for OVA<sup>258–76</sup> peptide generated as previously described were used (15). OVA peptide for all experiments was synthesized at the University of North Carolina Microprotein Sequencing and Peptide Synthesis Facility. The T cell hybridomas ( $10^5$ ) were incubated for 24 h with  $2 \times 10^5$  BMDCs or Type II cells (C57BL/6) and varying amounts of OVA<sup>258–276</sup> peptide in 96-well round-bottom plates in complete IMDM with 10% FCS, 25  $\mu$ M 2-ME, penicillin-streptomycin (100 U/ml), and gentamicin (20  $\mu$ g/ml). Murine IL-2 concentrations from supernatants were measured by ELISA (Pierce).

### CD4<sup>+</sup> T cell isolation

Spleens and lymph nodes were removed and minced, and cells were filtered through a 40- $\mu$ m strainer. Monocytes were removed by panning on plastic for 12–18 h and CD4<sup>+</sup> T cells were subsequently positively selected using paramagnetic microbeads (Miltenyi Biotec).

### Alloreactivity assay

CD4<sup>+</sup> splenocytes ( $10^5$ ) were incubated with allogeneic Type II cells or BMDCs from SP-C GFP mice for 3 days in 96-well round-bottom plates in complete DMEM with 10% FCS, 25  $\mu$ M 2-ME, penicillin-streptomycin (100 U/ml), and gentamicin (20  $\mu$ g/ml). One  $\mu$ Ci of [<sup>3</sup>H]thymidine (6.7 Ci/mmol; MP Biomedical) was added to each well and cells were incubated for another 24 h. Incorporated radioactivity was measured by means of scintillation as an indicator of proliferation.

### Anergy assays

DO11.10 CD4<sup>+</sup> T cells ( $5 \times 10^4$ ) were incubated for 3 days in the presence or absence of Type II cells ( $1.5\text{--}2 \times 10^5$ ; SP-C/GFP, BALB/c) and/or OVA<sup>323–339</sup> peptide (50  $\mu$ g/ml) in 96-well round-bottom plates in complete IMDM. Cells were washed, BMDCs ( $7.5\text{--}10 \times 10^4$ ) and fresh OVA peptide was added, and they were incubated for another 2 days, after which murine IL-2 concentrations from supernatants were measured by ELISA (R&D Systems). In assays wherein T cells were repurified on day 3, co-cultures were initially plated in 24-well plates. On day 3, T cells from each condition were positively selected with anti-CD4 microbeads (Miltenyi Biotec) and re-plated at equal numbers ( $1 \times 10^4$  cells/well) with BMDCs ( $1 \times 10^4$ ) and OVA (50  $\mu$ g/ml) in 96-well round-bottom plates.

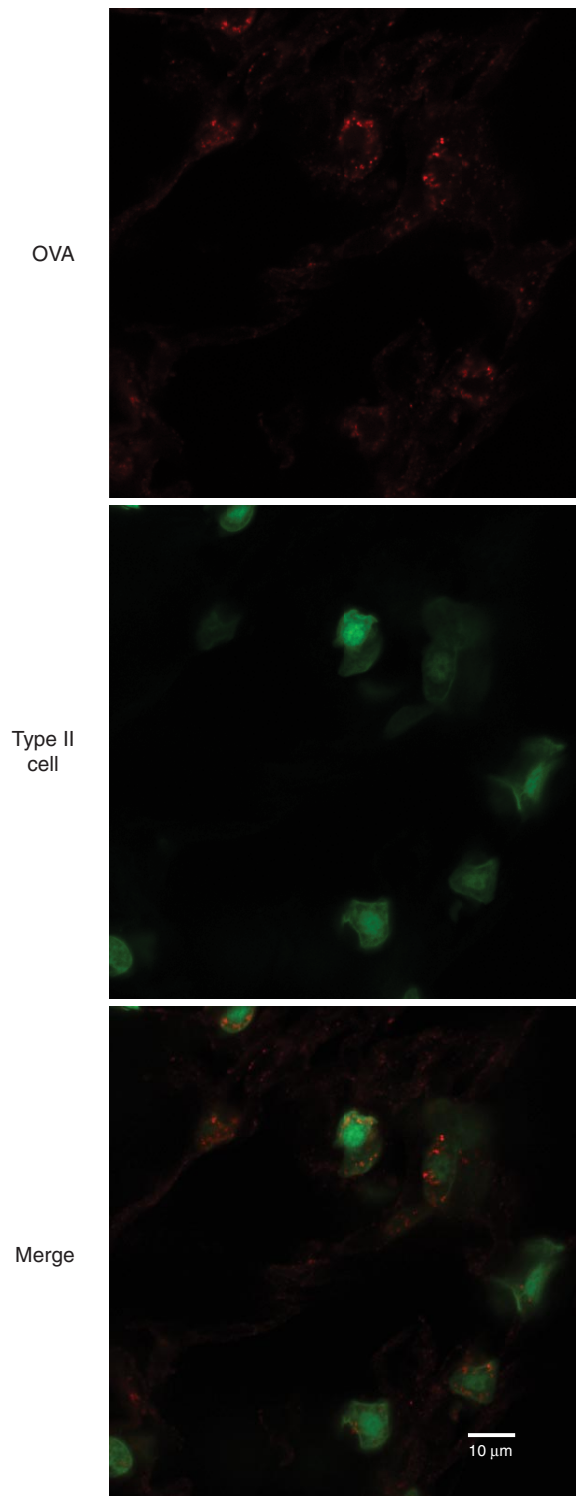


**FIGURE 1.** Type II cells take up Ag in vivo. Transgenic SP-C GFP mice, which express GFP in the Type II cells (green, center panel), were intratracheally instilled with fluorescently labeled OVA (red, top panel) and then, after 3 h, their lungs were fixed for confocal microscopy. Bottom panel is merge of top and center panels ( $\times 63$  magnification).

### Flow cytometry

Flow cytometry was performed at the Duke University Comprehensive Cancer Flow Cytometry and at the Human Vaccine Institute facilities. Lung digests from SP-C GFP mice were stained with biotinylated-MHC II, -CD80, -CD86, or -CD54 (ICAM-1) Abs (BD Pharmingen) and streptavidin-conjugated AlexaFluor 647 (Molecular Probes). FACS data were analyzed with

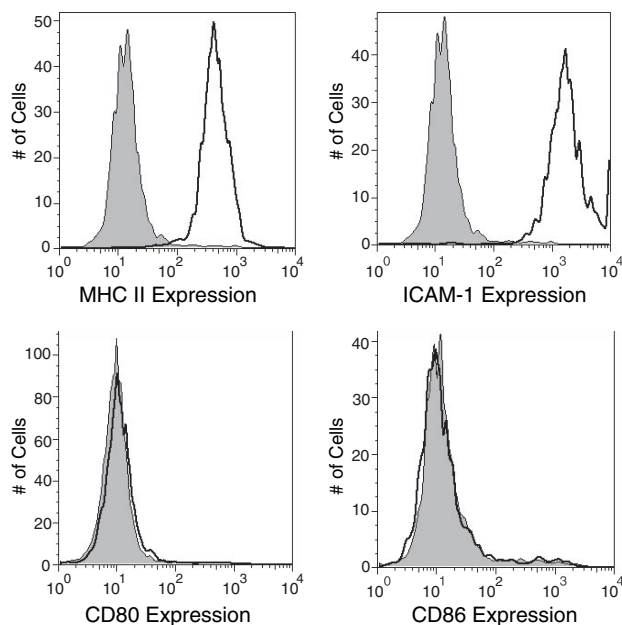


**FIGURE 2.** Type II cells express MHC II and ICAM-1 but not the costimulatory molecules, CD80 and CD86, for Ag presentation. Lung digests from SP-C GFP mice were stained for MHC II, CD80, CD86, and ICAM-1 and analyzed by flow cytometry. Expression of the molecules (open histograms) is shown for the GFP positive cells (Type II cells). Isotype or autofluorescence controls are shown as filled gray histograms.

FlowJo software (TreeStar). To determine Type II cell expression of molecules, analyses were done on high GFP gated cells.

#### OVA instillation

SP-C GFP mice were anesthetized with ketamine (100 mg/kg mouse) and xylazine (6 mg/kg) and intratracheally instilled with AlexaFluor 647-conjugated OVA (0.66 mg/50  $\mu$ l/mouse; Molecular Probes) in PBS. Controls were instilled with PBS alone. After 3–24 h, the mice were euthanized and their lungs lavaged with PBS. Lungs were fixed by tracheal inflation fixation with 4% paraformaldehyde in PBS and prepared for confocal microscopy. Thin slices of left lung were placed into chamber slides, covered in PBS, and photographed on a confocal Zeiss Axiovert 100M microscope.

#### Statistics

Data are expressed as mean  $\pm$  SEM. Statistical significance was tested with an unpaired Student's *t* test using Prism 4 program (GraphPad).

## Results

### Type II pneumocytes take up Ag in vivo

The primary function of the MHC II molecule is Ag presentation. Because Type II cells constitutively express MHC II, it has been hypothesized that they have the capacity to present Ag (7). To determine whether Type II cells take up the model Ag OVA in vivo, transgenic mice (SP-C GFP), which express GFP in Type II cells, were intratracheally instilled with fluorescently labeled OVA and examined at 3 h after the instillation. Fluorescently labeled OVA colocalized within the GFP positive Type II cells (Fig. 1). Z-stacks were used to confirm that the fluorescent OVA was internalized in the Type II cells. This finding demonstrates that Type II cells have the capacity to take up Ag in our in vivo mouse model.

### Type II pneumocytes express MHC II but not the costimulatory molecules CD80 and CD86

Because the Type II cells are capable of Ag uptake, we explored whether the Type II cells in our model express the necessary mol-

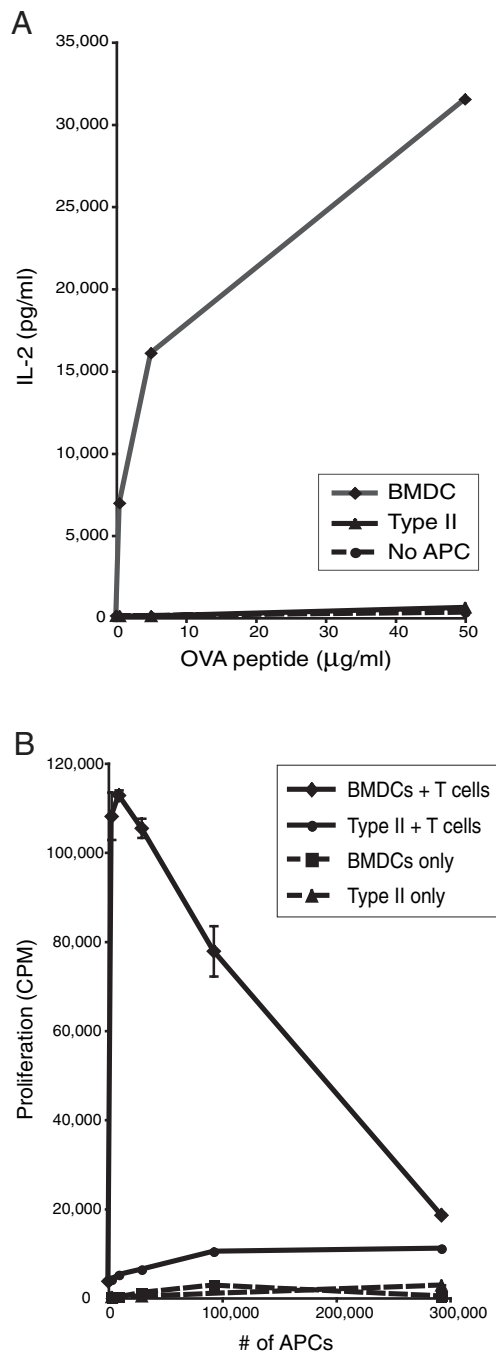


**FIGURE 3.** Type II cells are poor stimulators of T cell activation. *A*, T cell hybridomas ( $10^5$ ) were incubated for 24 h with  $2 \times 10^5$  Type II cells or BMDCs and OVA, and then IL-2 concentrations were measured by ELISA. *B*,  $10^5$  T cells were incubated with allogeneic Type II cells or BMDCs for 3 days, tritiated thymidine was added and incubation continued for another 24 h. Cells were harvested and incorporated radioactivity was measured as indicator of T cell proliferation. *A*, Data shown are representative of three independent experiments done in duplicate. *B*, Results are indicative of three similar independent experiments done in triplicate. Values are mean of triplicate  $\pm$  SE.

ecules for Ag presentation and T cell activation. The expression of MHC II and the costimulatory molecules, CD80 and CD86, on murine Type II cells was analyzed using flow cytometry. In this study, we confirmed the previously reported (7) constitutive surface expression of MHC II and showed that neither CD80 nor CD86 were expressed at detectable levels (Fig. 2). Additionally,

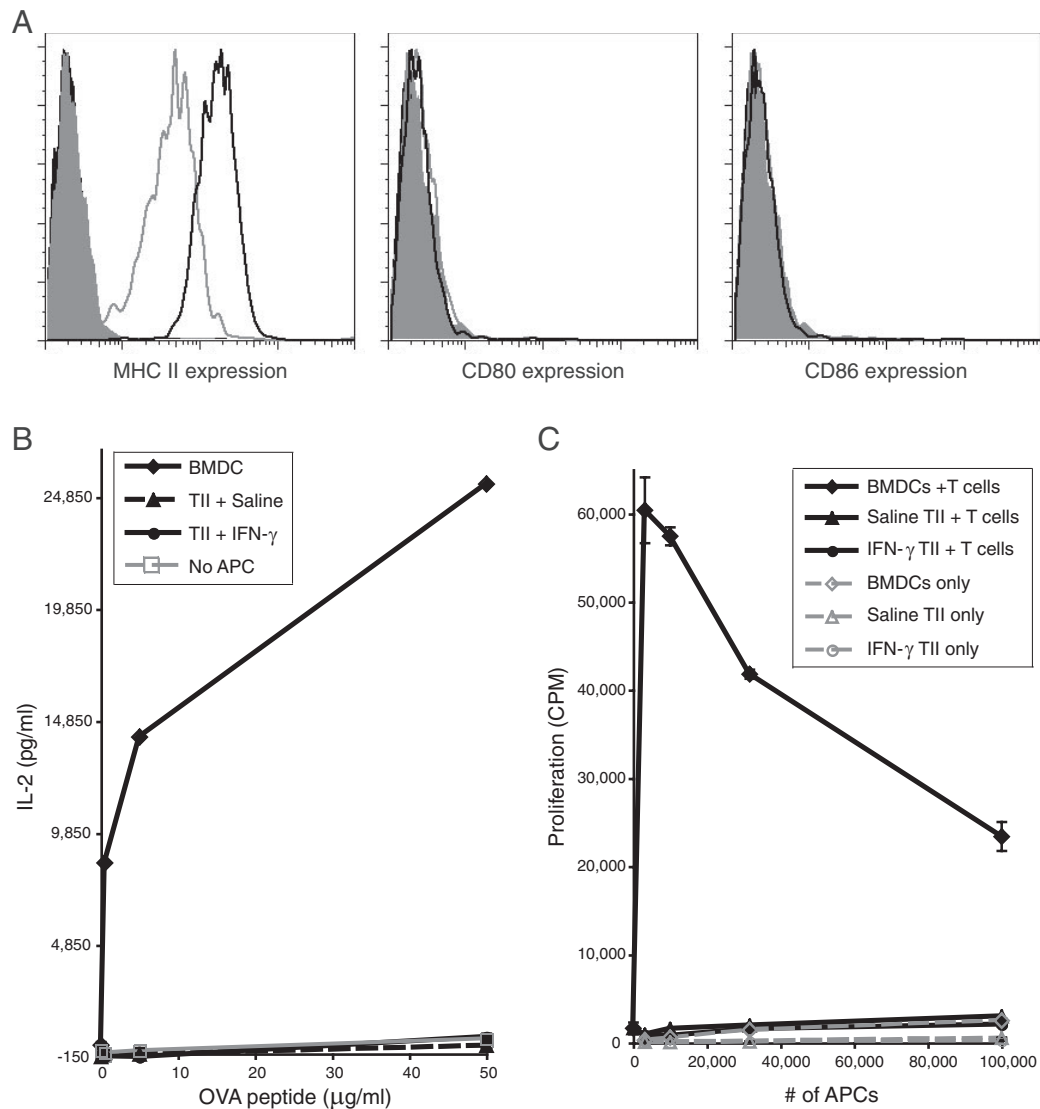


**FIGURE 4.** Although IFN- $\gamma$  treatment of Type II cells increased MHC II, Type II cells were still poor stimulators of T cell activation. **A**, Type II cells were isolated from SP-C GFP mice treated for 3 days with IFN- $\gamma$  (in black) or saline (in gray). Isolated cells were analyzed for expression of MHC II, CD80, and CD86 (open histograms). Isotype (for MHC II) or autofluorescence (for CD80 and CD86) controls are shown as filled histograms. **B**,  $10^5$  T cell hybridomas were incubated with  $2 \times 10^5$  IFN- $\gamma$  or saline treated Type II cells (TII) or BMDCs for 24 h. Then, IL-2 was measured in the supernatants by ELISA. **C**,  $10^5$  T cells were incubated with allogeneic IFN- $\gamma$  or saline treated Type II cells or BMDCs for 3 days. Then, tritiated thymidine was added and the cells were incubated for another 24 h. Incorporated radioactivity was measured as indicator of T cell activation and proliferation. **B**, Shown is a representative experiment of three independent experiments done in duplicate. **C**, Results are indicative of two independent experiments done in triplicate. Values are mean of triplicate  $\pm$  SE.

we found ICAM-1 on the Type II cells, which has been reported to act as an accessory molecule for Ag presentation to T cells (16). We also analyzed for the expression of additional costimulatory molecules, including CD40 and inducible T cell costimulator ligand (ICOSL), and the inhibitory molecules, programmed death-1 ligands 1 and 2 (PD-L1 and 2). We found no detectable levels of any of these molecules on the isolated Type II cells (data not shown).

#### *Type II pneumocytes are poor stimulators of T cell activation compared with BMDCs*

Costimulatory molecules are essential for effective activation of T cells. Because Type II cells do not express detectable levels of CD80 and CD86, we hypothesized that they would not be able to induce a robust activation of T cells. To test this hypothesis, Type II cells were examined for their ability to present the model Ag,

OVA, and to activate T lymphocytes. BMDCs were used as the positive control APCs as they should effectively activate T cells. Purified Type II cells or BMDCs were incubated with OVA peptide and T<sub>H</sub>-hybridomas, specific for the OVA peptide. After 24 h, IL-2 concentrations were measured by ELISA as an indicator of T cell activation. As shown in Fig. 3A, negligible levels of IL-2 were secreted from T cells incubated with the Type II cells or by T cells incubated with OVA alone in the absence of any APCs (no APC). In contrast, in the presence of OVA, BMDCs stimulated secretion of high concentrations of IL-2 by the T cells.

To confirm further the inability of Type II pneumocytes to effectively stimulate T cell activation, we tested whether Type II cells could activate allogeneic CD4<sup>+</sup> T cells. Type II cells or BMDCs were incubated with allogeneic CD4<sup>+</sup> T cells for 3 days, after which time [<sup>3</sup>H]thymidine was added. After ~24 h, cells were harvested and [<sup>3</sup>H]thymidine incorporation was measured as

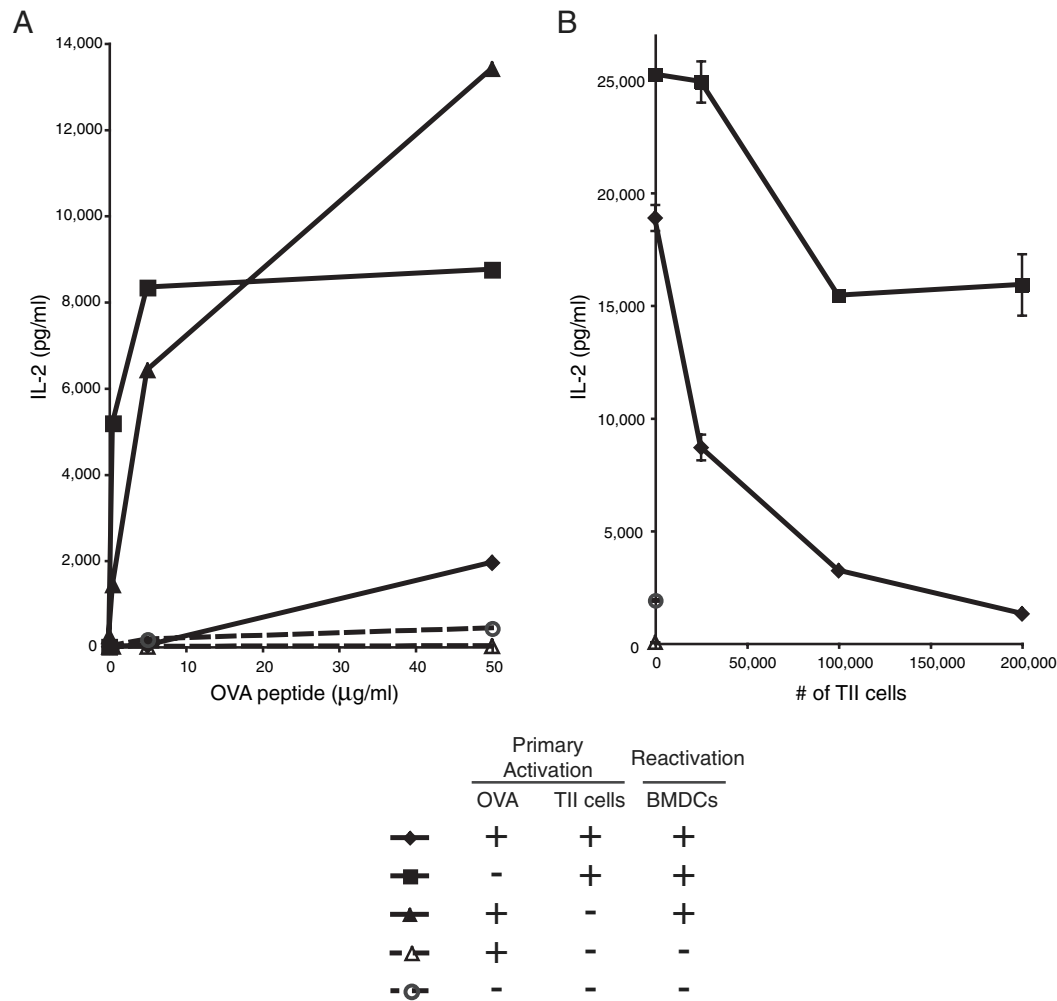


**FIGURE 5.** Type II cells suppress subsequent T cell activation in an Ag-dependent fashion. *A*, Five  $\times 10^4$  T cells were incubated with  $2 \times 10^5$  Type II cells (TII) and/or varying concentrations of OVA (primary activation) for 3 days, then washed. To reactivate,  $7.5 \times 10^4$  BMDCs and 50  $\mu\text{g/ml}$  fresh OVA were added, and the cells were cultured for another 2 days. IL-2 was measured from supernatants by ELISA. *B*, Type II cells dose dependently suppress subsequent T cell activation in an Ag-specific manner. Five  $\times 10^5$  T cells were incubated with increasing numbers of Type II cells with or without 50  $\mu\text{g/ml}$  OVA for 3 days. Cells were then washed and BMDCs and fresh OVA was added to the cultures. Cells were incubated another 2 days, then IL-2 was measured as readout for T cell activation. *A*, Shown is representative of three similar experiments done in duplicate. *B*, Results are indicative of three independent experiments done in triplicate. Values are mean of triplicate  $\pm$  SE.

an indicator of T cell activation and proliferation. Fig. 3*B* shows that Type II cells were poor stimulators of alloreactivity in T cells, whereas BMDCs were potent stimulators of allogeneic T cell proliferation, even at low APC to T cell ratios. Together, the data show that Type II cells are incapable of stimulating detectable T cell activation.

*Type II pneumocytes from IFN- $\gamma$ -stimulated mice are still poor stimulators of T cell activation*

IFN- $\gamma$  has been shown to up-regulate expression of MHC II on Type II cells (7). To determine whether the inability of Type II cells to effectively activate T cells is due to insufficient MHC II expression, the Type II cells were tested for their ability to stimulate T cells after their MHC II levels were up-regulated by i.v. injection of IFN- $\gamma$  over a course of 3 days. Fig. 4*A* shows that upon IFN- $\gamma$  treatment, MHC II expression was increased on the surface of Type II cells, but CD80 and CD86 expression was still undetectable. Furthermore, after IFN- $\gamma$  treatment, Type II cells were still poor stimulators of T cell activation (Fig. 4*B*). Regardless of treatment, the Type II cells stimulated negligible levels of IL-2 production by the T cells compared with the negative control with

T cells and OVA alone (no APC). Type II cells from IFN- $\gamma$  or saline control-treated mice were also tested for their capacity to activate allogeneic CD4 $^+$  T cells. Consistent with the previous data, IFN- $\gamma$ -induced up-regulation of MHC II did not affect the ability of Type II cells to activate the allogeneic T cells (Fig. 4*C*). Together, these data demonstrate that the level of MHC II expression does not affect the Type II cell's ability to activate T cells, which is consistent with the evidence that they do not possess the proper costimulatory molecules necessary for complete T cell activation (10, 17, 18).

*Type II pneumocytes suppress subsequent T cell activation in an Ag-specific fashion*

Because Type II cells express MHC II, but not the costimulatory molecules CD80 and CD86, we hypothesized that Type II pneumocytes may tolerize T cells. This hypothesis was tested by co-incubating Type II cells and DO11.10 T lymphocytes with or without OVA peptide for 3 days. The cells were then washed and BMDCs and fresh OVA peptide were added, and 2 days later, IL-2 concentrations in the supernatants were measured to determine whether the pretreated T cells could be activated. T cells that were

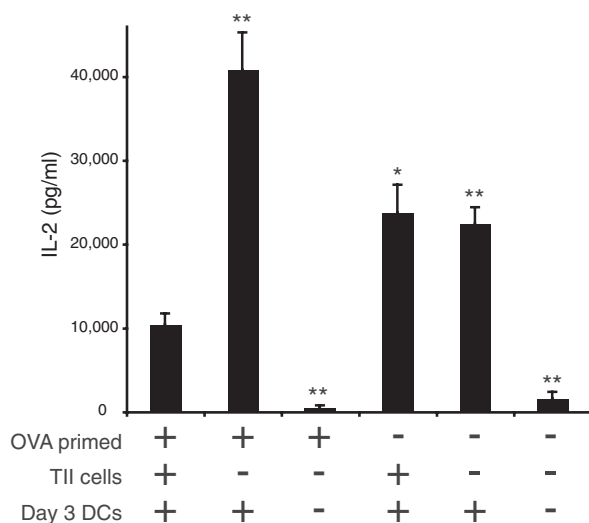


**FIGURE 6.** Type II cell-induced suppression persists in subsequent T cell activation even in the absence of further Type II cell stimulus. Three  $\times 10^5$  T cells were cocultured in the presence or absence of  $6 \times 10^5$  Type II cells (TII) with or without 50  $\mu\text{g/ml}$  OVA for 3 days. Then, T cells were repurified, plated at equal numbers ( $10^4$ ), and stimulated with  $10^4$  BMDCs and OVA. After 2 days, supernatants were collected and IL-2 was measured by ELISA. Results are expressed as a mean  $\pm$  SE of three independent experiments done in triplicate. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  vs T cells preincubated with Type II cells and OVA.

preincubated with OVA peptide and Type II cells and then activated with BMDCs and additional OVA secreted lower levels of IL-2 compared with T cells treated under the same conditions with the exception of the addition of Type II cells (Fig. 5A). The high levels of IL-2 secreted from the T cells preincubated in absence of OVA but in the presence of Type II cells demonstrate that the reduced IL-2 secretion seen in the T cells preincubated in the presence of OVA and Type II cells is not due to nonspecific suppression of T cell function due to Type II cell cocubation. Rather, it demonstrates that the Type II cells are capable of suppressing T cells in an Ag-specific manner. The level of IL-2 secretion from the T cells incubated with OVA, Type II cells, and BMDCs above background IL-2 secretion from T cells incubated with OVA alone may be due to those T cells that were not suppressed sufficiently during the initial priming. Fig. 5B shows that the Type II cells dose-dependently suppress subsequent T cell activation in an Ag-specific manner. Increasing numbers of Type II cells in the OVA-prestimulated T cell cocultures resulted in greater suppression. Additionally, we analyzed levels of the immunosuppressive cytokine TGF- $\beta$  in the supernatants after the preincubation of T cells with or without Type II cells in the presence or absence of OVA. We found no significant difference in total TGF- $\beta$  (active and latent) concentrations between T cells cocultured with or without Type II cells (data not shown). Active TGF- $\beta$  was not detected in any of the supernatants tested (detection limit  $\sim 47$  pg/ml; data not shown). These data indicate that TGF- $\beta$  may not contribute significantly to the Type II cell-induced T cell suppression.

Upon induction of tolerance, a T cell is "intrinsically functionally inactivated" (11). Thus, to test whether the observed T cell unresponsiveness requires the continuous presence of Type II cells, the T cells were removed from the Type II cell coculture on day 3 and repurified before stimulation with BMDCs and OVA. As shown in Fig. 6, T cells initially stimulated with Type II cells and OVA are less responsive upon subsequent activation than T cells incubated alone with OVA or the T cells that were not preincu-

bated with OVA in the presence or absence of Type II cells. This finding demonstrates that the Ag-specific Type II cell-induced T cell suppression is not dependent on constant Type II cell contact or presence and that the suppression of T cells is not through an indirect effect of the Type II cells on the BMDCs capacity to stimulate T cells.

## Discussion

Because MHC II expression is predominantly found only on professional APCs that function to present Ag to T cells, the discovery that Type II cells constitutively express MHC II suggested that Type II cells may function in the immune response (7). Currently, the role of the Type II cell in the immune system is not clearly understood. In this study, we show that Type II cells isolated from the naive lung can tolerize T cells to an Ag that serves as a model of the innocuous Ags to which the lungs are constantly exposed. We demonstrated that Type II cells suppress T cells from future activation in an Ag-specific fashion.

We also demonstrated that, although Type II cells express MHC II, they could not effectively activate T cells with specific Ag using an OVA model or even by alloreactivity. BMDCs were capable of generating a robust T cell activation using both assays. With increasing concentrations of OVA, BMDCs elicited higher levels of IL-2 secreted by the T cells. The BMDCs were also capable of stimulating alloreactivity, even at numbers as low as  $3 \times 10^3$ . At very high ratios of BMDCs to T cells, incorporated [ $^3\text{H}$ ]-thymidine levels were reduced compared with those measured at lower ratios of BMDCs to T cells, possibly as a consequence of the high level of proliferation resulting in medium depletion or acidification. Comparatively, Type II cells were weak stimulators of T cell activation, inducing only low levels of IL-2 secretion or T cell proliferation above the control levels observed with T cells alone. This inability to potently activate T cells may be explained by the lack of costimulatory molecule expression on Type II cells. For this study, the Type II cells were plated directly on plastic with T cells immediately after isolation. Because Type II cells are often cultured on an extracellular matrix, we also tested whether Type II cells cultured on a matrix (70/30 by volume) of Matrigel and collagen, as previously described (19), activated T cells by alloreactivity. Consistent with the data in Fig. 3B, the Type II cells cultured on Matrigel were poor stimulators of T cell activation compared with BMDCs, inducing only low levels of T cell proliferation above the controls. Thus, Type II cells, whether plated on plastic or extracellular matrix, are unable to effectively activate T cells.

Although we showed that Type II cells did not significantly activate naive T cells in our system, others have shown that Type II cells are capable of stimulating some effector or memory T cells (20). These findings are consistent with the possibility that, although Type II cells cannot effectively activate naive T cells, Type II cells may still be capable of activating some T cells in the effector phase of an immune response or in a secondary (memory) immune response because previously activated T cells are less dependent on costimulation. Additionally, Qian et al. (21) showed by immunohistochemistry that Type II cells from mice and the A549 Type II-like cell line both express ICOSL, which is a costimulatory molecule that can stimulate activated and memory T cells (22). It is important to note that, in the study of Qian et al. (21), Type II cells were not identified by expression of a specific marker (e.g., SP-C) and therefore, the identity of the cells as Type II cells cannot be confirmed as other cells types, especially since alveolar macrophages and other leukocytes, can resemble Type II cells at the light microscope level. We were unable to detect ICOSL on isolated Type II cells by flow cytometry, but we found that the enzyme Dispase used for Type II cell isolation degraded ICOSL,

suggesting that if ICOSL is expressed by mouse Type II cells, it may be degraded during the isolation process. However, because ICOSL does not activate naive T cells due to the lack of its receptor (ICOS) expression on naive T cells, Type II cells may play dual roles in the immune response. In the healthy lung, Type II cells may tolerize naive or resting T cells to the plethora of non-pathogenic particulates and potential allergens, but in the infected or inflamed lung, Type II cells may be capable of aiding in the activation of T cells in the effector phase of the immune response.

Because stimulation of naive T cells requires Ag in the context of MHC II in the presence of costimulatory molecules, we hypothesized that Type II cells, which do not express detectable levels of CD80 or CD86, tolerize T cells. Our data show that Type II cells isolated from the normal lung suppress subsequent T cell activation in an Ag-dependent manner, which is indicative of tolerance. In addition, Type II cells dose-dependently suppressed future T cell activation in an Ag-dependent fashion. There is also some detectable Ag-independent suppression by the Type II cells, although this may be attributable to medium acidification and depletion of medium nutrients caused by rapid T cell proliferation that occurred in the presence of the higher numbers of Type II cells and BMDCs. The suppression of T cells by Type II cells did not require the continuous presence of Type II cells, because upon the removal of the Type II cells, the T cells were still hyporesponsive to subsequent activation with BMDCs. This intrinsically suppressed state is a hallmark of tolerance (11). The T cells that were initially incubated with Type II cells and OVA secreted significantly less IL-2 compared with the T cells incubated alone with OVA or the T cells that were not preincubated with OVA in the presence or absence of Type II cells. The highest levels of IL-2 secretion were seen in the condition with the T cells that were initially primed alone with OVA, which may be due to the activation of these T cells during the initial OVA incubation by the few contaminating APCs in the purified T cell preparation, because the purified T cells were generally only ~95–98% pure.

Although we found that Type II cells do not express detectable levels of CD80 or CD86, they do express high levels of ICAM-1. The expression of the accessory molecule, ICAM-1, along with MHC II in the absence of CD28 costimulation has been shown to be sufficient to induce tolerance in naive T cells (23). Thus, Type II cells appear to possess the necessary molecules for inducing tolerance in naive T cells. Moreover, a recent study provided evidence that not only effector T cells but also naive T cells migrate through nonlymphoid organs, especially the lung, and proposed that the purpose of the migration may be for peripheral tolerance induction (24). Seen in this aspect, it is intriguing to speculate that Type II cells may participate in the induction of peripheral tolerance.

In addition to analyzing the expression of costimulatory molecules on Type II cells, we also tested for the expression of the inhibitory ligands PD-L1 and PD-L2, because the PD-1-PD-L inhibitory signaling pathway is implicated in suppressing T cell responses and promoting T cell tolerance (25). The expression of PD-L1 and PD-L2 has not been reported on Type II cells *in vivo* or on primary Type II cells, although it has been reported that PD-L1 and very low levels of PD-L2 are expressed on the A549 cell line (26). We were not able to detect PD-L1 or PD-L2 expression on isolated Type II cells, however we found that the enzyme Dispase used for Type II cell isolation degraded PD-L1 and PD-L2 suggesting that if they were expressed by mouse Type II cells, they may be degraded during the isolation process. Because analysis of Type II cell function *in vitro* requires that the cells be isolated by enzymatic digestion, we cannot exclude the possibility that Type II cells *in situ* express PD-L1 or PD-L2. Nevertheless, our data

do support the conclusion that the Type II cell tolerizing mechanism we have observed *in vitro* is independent of PD-L1 or PD-L2.

OVA peptide was used for our Ag presentation assays to circumvent the problems with determining Ag processing efficiency in Type II cells *in vitro*. Although we did not demonstrate that Type II cells have the capacity to process Ag for presentation, others have shown that Type II cells have the ability to take up and process Ag. In A549 cells, a Type II-like cell line, Ag uptake and trafficking was shown to follow a class II endocytic pathway in which Ag colocalized with intracellular MHC II (27). *In vivo* uptake of HRP by rat type II cells has also been demonstrated (28). We additionally confirmed that Type II cells *in vivo* could internalize OVA protein. Finally, Debbabi et al. (20) showed that Ag processing was necessary for the Type II cells to stimulate the preactivated T cells using an Ag pulse-fix method. Type II cells pulsed with protein Ags before fixing were capable of stimulating the T cells, but those fixed before being incubated with Ags could not stimulate the T cells.

In this report, we demonstrate that Type II cells express MHC II but do not express CD80 or CD86. It is important to note, though, that there are conflicting studies in the literature regarding Type II cell expression of CD80 and/or CD86. For example, Zissel et al. (29) found by flow cytometry that CD80 and CD86 were expressed on human Type II cells from tumor-free lung sections from tumor patients. However, the parameters for defining percent positive for CD80 or CD86 was not defined and it appears that nonspecific binding was not assessed using isotype controls. In contrast, a study by Cunningham et al. (9), which also used isolated primary human Type II cells but included nonspecific Ab controls, found no CD80 or CD86 expressed on human Type II cells by flow cytometry. Other studies reporting the expression of CD80 and/or CD86 on Type II cells did not use primary isolates of Type II cells, but rather used the carcinoma-derived A549 cell line (30, 31), which is sometimes used as a Type II-like cell line but is not truly representative of Type II cells of the alveolar space (32). Other reports used cultures of respiratory epithelial cells that may contain Type II cells, but also likely includes other epithelial cells that may confound their results (31). Thus, it is important to consider these issues when comparing our studies with those referenced above.

To validate further our data showing the expression of MHC II and lack of CD80 and CD86, we confirmed that the enzymatic isolation method used for obtaining primary Type II cells has no effect on the cell surface expression of CD80 and CD86. We incubated splenocytes or BMDCs with Dispase enzyme for 1 or 2 h and analyzed them for cell surface protein expression of a variety of markers by flow cytometry. We found that although Dispase treatment resulted in the complete loss of cell surface ICOSL and a greater than 80% loss of cell surface PD-L1 and 2 (as discussed above), it only minimally affected surface expression of CD80, CD86, CD54, and CD40 (data not shown). MHC II was reduced to a slightly greater extent (30–40% reduction). Thus, the lack of CD80 or CD86 on primary Type II cells does not appear to be a consequence of the enzymatic isolation method.

Positioned in the alveolus at the barrier between the outside gases and the body's vital blood supply, Type II alveolar epithelial cells are at the optimal position for defending the lungs and body from the continuous barrage of pathogenic and nonpathogenic inhaled Ags. During a pulmonary infection, Type II cells may be able to help defend the lungs from pathogenic Ags by stimulating T cells in the effector phase of the immune response. We propose that in the healthy lung, Type II cells protect the lungs from unnecessary inflammation by tolerizing T cells to the numerous potentially innocuous Ags to which the lungs are daily exposed.

Thus, studying this mechanism of suppression may aid in the understanding of pulmonary inflammatory diseases, such as allergies or asthma, which may occur when there is a break in tolerance.

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## Disclosures

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