Immunobiology 220 (2015) 1240–1245

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Cell-contact dependent inhibition of monocytes by airway epithelial cells and reversion by infection with *Respiratory Syncytial Virus*

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ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 17 June 2015
Accepted 22 June 2015
Available online 27 June 2015

Keywords:
Airways
Epithelial cells
Infection
Innate Immunity
Monocytes
Mucosal Immunology

ABSTRACT

Airway epithelial cells (AEC) are the first line of defense against airborne infectious microbes and play an important role in regulating the local immune response. However, the interplay of epithelial cells and professional immune cells during both homeostasis and infection has only been partially studied. The present study was performed to determine how bronchial epithelial cells affect the activation of monocytes. Under healthy conditions, AECs were shown to inhibit reactivity of monocytes. We hypothesized that upon infection, monocytes might be released from inhibition by AECs. We report that direct contact of monocytes with unstimulated BEAS2B epithelial cells results in inhibition of TNF secretion by activated monocytes. In addition to the known soluble modulators, we show that cell contacts between epithelial cells and monocytes or macrophages also contribute to homeostatic inhibitory actions. We find AECs to express the inhibitory molecule PD-L1 and blockade of PD-L1 results in increased secretion of pro-inflammatory cytokines from monocytes. Contrary to the inhibitory activities during homeostasis, epithelial cells infected with *Respiratory Syncitial Virus (RSV)* induce a significant release of inhibition. However, release of inhibition was not due to modulation of PD-L1 expression in AECs. We conclude that airway epithelial cells control the reactivity of monocytes through direct and indirect interactions; however tonic inhibition can be reverted upon stimulation of AECs with RSV and thereof derived molecular patterns. The study confirms the important role of airway epithelial cells for local immune reactions.

1. Introduction

Airway epithelial cells (AEC) represent the first line of defense against infectious, airborne pathogens. Formation of tight junctions, mucociliary clearance and secretion of anti-microbial peptides make up the epithelial defense function. Moreover, it has been recognised over the last years, that AECs use pattern recognition receptors to actively sense infectious organisms and respond with an inducible program towards microbial contact (Parker and Prince, 2011). Thus, AECs participate in shaping local immune responses (Hammad and Lambrecht, 2011). However, pattern recognition receptors cannot discriminate pathogenic from non-pathogenic microorganisms, rather, they recognize conserved microbial structures. Thus, recognition at non-sterile mucosal surfaces, including the airways, must be regulated in an organ-specific manner. With these limitations in mind, Eyal Raz proposed a concept of organ-specific immune responses in which the organ-specific microenvironment regulates immune responses (Raz, 2007). Non-professional immune cells, including epithelial cells and stroma cells, are identified as important regulators of immune responses. This concept makes two assumptions: pattern recognition sensitivity has to be controlled at mucosal surfaces and organ-specific cells instruct local immune responses.

Indeed, sensitivity and activation threshold of pattern recognition receptors have been shown to be regulated within the airways (Guillot et al., 2004; Mayer et al., 2007; Shuto et al., 2006). AECs also modulate the reactivity of local immune cells. T-cell activation and innate immune reactions are controlled through the secretion of immune-modulatory factors including TSLP, IL-10, nitric oxide,

Abbreviations: AEC, airway epithelial cell; PD-L, programmed death-ligand; PGE, prostaglandin; RSV, *Respiratory Syncitial Virus*; TSLP, thymic stromal lymphopoietin.

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http://dx.doi.org/10.1016/j.imbio.2015.06.017
0171-2985/

LL37, TGF-β, prostaglandin E_2 and glucocorticoids (Bingisser and Holt, 2001; Kandler et al., 2006; Schmidt et al., 2011; Weitnauer et al., 2014). Moreover, local immune cells are found in close proximity to airway epithelial cells (Jahnsen et al., 1998), suggesting cell–cell interactions might also contribute to modulatory functions. The adhesion molecule ICAM-1 is expressed on epithelial cells and could interact with leukocyte function-associated molecule-1 on T-cells (Atsuta et al., 1999). Similarly, E-cadherin could interact with CD103 on intraepithelial T cells (Smyth et al., 2007) or dendritic cells (Sung et al., 2006).

Among potential candidates for interactions of airway epithelial cells with immune cells are members of the B7 family, known ligands for costimulatory and inhibitory receptors on T-cells. A variety of B7 homologues have been identified, with PD-L1 (B7-H1) (Dong et al., 1999) and PD-L2 (B7-H3) (Tseng et al., 2001) triggering the negative regulatory receptor PD-1 (Freeman et al., 2000). Both PD-L1 and PD-L2 have been found on airway epithelial cells (Heinecke et al., 2008; Kim et al., 2005) and PD-1 is not only expressed on lymphocytes but also on myeloid innate immune cells (Huang et al., 2009; Yao et al., 2009). Whereas regulation of PD-L1 and PD-L2 has been described during infection with RSV (Stanciu et al., 2006; Telcian et al., 2011) or rhinovirus (Heinecke et al., 2008), whether those ligands contribute to steady-state-interactions of airway epithelial cells with innate immune cells has not been tested. Moreover, it is unclear if the tonic inhibition of immune cells by airway epithelial cells observed in homeostasis can be reverted upon microbial stimulation.

In this report, we test the hypothesis that PD-L1 and PD-L2 are expressed on AECs and contribute to inhibition of monocytes. Moreover, we speculate that during microbial stimulation, negative regulation by AECs can be overcome in order to achieve appropriate pro-inflammatory reactions.

2. Material and methods

2.1. Reagents and antibodies

RPMI 1640 was obtained from Biochrom (Berlin, Germany) and supplemented with 10% FCS (Gibco/Life Technologies, Carlsbad, USA) and penicillin and streptomycin (PAA, Coelbe, Germany). LPS from *Salmonella minnesota* was a kind gift by U. Seydel (Research Center Borstel, Germany), poly I:C was from Sigma–Aldrich (Schnelldorf, Germany) and Pam_3CSK_4 was obtained from Invivogen (Toulouse, France). TNF ELISA kit was purchased from BD Biosciences (Heidelberg, Germany). Antibodies for FACS analysis (PD-L1 APC and PD-L2 PE, isotype controls) and neutralizing PD-L1 and PD-L2 antibodies were from eBioscience (Frankfurt, Germany). CFDA-SE/CFSE was purchased from Invitrogen (Karlsruhe, Germany).

2.2. Cell culture and epithelial cell conditioned medium (ECCM)

Human bronchial epithelial cell lines BEAS-2B and A549 were maintained essentially as described previously (Mayer et al., 2007) in DMEM/10% FCS. For preparing epithelial cell conditioned medium, (ECM) 2.5×10^4 cells were seeded in 96-well plates and cultivated for 48 h prior to harvesting the supernatant.

2.3. Isolation of $CD14^+$ monocytes and generation of macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood of healthy volunteer donors by standard Ficoll–Paque density gradient centrifugation and washed three times. Blood sampling was approved by the local ethic committee. For purification of monocytes, $CD14^+$ cells were positively selected by magnetic associated cell sorting (AutoMACS, program: possel; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. Purity of cell populations was verified by FACS staining and exceeded 95%. Human monocyte-derived macrophages were generated by incubating CD14-positive monocytes with recombinant hMCSF (50 ng/ml, R&D, Abington, UK) for 8 days.

2.4. Stimulation of epithelial cells

Direct coculture: freshly split airway epithelial cells were harvested and counted. 10^4 or 10^5 cells were seeded in each well of a 24 well cell culture plate (Cellstar, Greiner, Frickenhausen, Germany). Cells were left to adhere for 6 h in DMEM/10% FCS. Where indicated, cells were stimulated with TLR ligands, infected with RSV overnight or left unstimulated. The next day cells were washed and 10^5 purified $CD14^+$ monocytes were added to each well containing epithelial cells. After two hours of co-incubation with epithelial cells, monocytes were stimulated with 10 ng/ml LPS overnight. Incubation in ECCM: monocytes were stimulated in the presence of 25% v/v ECCM (100 μl in total volume of 400 μl) obtained from 48 h old epithelial cell cultures. As controls, monocytes were cultured without addition of epithelial cells or their supernatant and left unstimulated (negative control) or were stimulated with LPS (positive control).

2.5. Blocking antibodies

Anti PD-L1 or anti PD-L2 antibodies or the isotype control were added to the cocultures at a concentration of 10 μg/ml.

2.6. Transwell experiments

10^5 BEAS-2B or A549 cells were seeded in 24-well cell culture plates overnight (bottom). The next day, 10^5 human monocytes or monocyte-derived macrophages were seeded into the transwell inserts (top) or added directly on the epithelial cells. Cells were stimulated with 10 ng/ml LPS overnight.

2.7. Flow cytometry

Cells were analyzed on a FACS Canto (BD, Heidelberg, Germany). Therefore, cells were washed in PBS and stained directly with FITC, PE or APC labeled antibodies against PD L1, PD-L2, CD14 and CD4. Overlays were done with WinMDI Vs. 2.9.

2.8. ELISA

Cell free supernatants were harvested and analyzed for TNF secretion by a commercially available ELISA kit (OptEIA; BD) following the recommendations of the manufacturer.

2.9. RSV

RSV strain A2 was propagated in Hep2 cells, maintained in RPMI supplemented with 5% heat-inactivated fetal bovine serum (FBS). Infected cells were incubated at 37 °C. Four–seven days after infection, infected cells were collected by scraping and were either frozen at −80 °C or used at a dilution of 1:10 to infect more cells for up to two infection cycles. Virus titres were determined by plaque assay on Hep2 cells. Briefly, tenfold serial dilutions in RPMI with 5% FBS were added to 70–80% confluent Hep2 monolayers in 96-well tissue-culture plates. Cells were incubated for 7 days at 37 °C, and HRSV plaques were determined by 2% crystal violet staining. Viral titres were expressed in p.f.u. per milliliter. BEAS-2B cells (70% confluent) were infected at a multiplicity of infection (MOI) of 1.0 with RSV strain A2.