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Research paper

Autocrine GABA signaling distinctively regulates phenotypic activation of mouse pulmonary macrophages

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ABSTRACT

In response to micro-environmental cues such as microbial infections or T-helper 1 and 2 (T_H1 and T_H2) cytokines, macrophages (Mϕs) develop into M1- or M2-like phenotypes. Phenotypic polarization/activation of Mϕs are also essentially regulated by autocrine signals. Type-A γ-aminobutyric acid receptor ($GABA_AR$)-mediated autocrine signaling is critical for phenotypic differentiation and transformation of various cell types. The present study explored whether $GABA_AR$ signaling regulates lung Mϕ (LMϕ) phenotypic activation under M1/T_H1 and M2/T_H2 environments. Results showed that $GABA_AR$ subunits were expressed by primary LMϕ of mice and the mouse Mϕ cell line RAW264.7. The expression levels of $GABA_AR$ subunits in mouse LMϕs and RAW264.7 cells decreased or increased concurrently with classical (M1) or alternative (M2) activation, respectively. Moreover, activation or blockade of $GABA_ARs$ distinctively influenced the phenotypic characteristics of Mϕ. These results suggested that microenvironments leading to LMϕ phenotypic polarization concurrently modulates autocrine GABA signaling and its role in Mϕ activation.

1. Introduction

Macrophages (Mϕs) exist in almost all tissues of the body where they play critical roles in mediating tissue homeostasis as well as primary innate immune responses [1]. Mϕs in the lung (LMϕs) including airway/alveolar and interstitial Mϕs (AMϕs and IMϕs respectively) are the predominant immune cells in pulmonary tissues under normal conditions [2]. Previous studies indicated that LMϕs reacting to environmental cues, such as inhaled pathogens and the cytokine milieu, develop into either classically-activated (M1) or alternatively-activated (M2) Mϕs [3]. For example, LMϕs reacting to the Gram-negative bacterial endotoxin lipopolysaccharide (LPS) and/or the T helper 1 (T_H1) cytokine interferon-gamma (INFγ) polarize to the pro-inflammatory M1 phenotype characterized by upregulated inducible nitric oxide synthase (iNOS) expression and tumor necrosis factor alpha (TNFα) secretion. Alternatively, following exposure to allergen or microbes inducing T helper 2 (T_H2) cytokines, such as interleukin-4/13 (IL-4/13), LMϕs shift to “anti-inflammatory” and “pro-wound healing” M2a-d phenotypes, which are generally characterized by increased expression of arginase-1 and secretion of IL-10 [4]. LMϕs strategically coordinate innate immune responses by adopting different functional phenotypes. They do so through phagocytosis of invading bacteria and injured tissue cells during pulmonary inflammation. Concurrently with these activities, Mϕ secret specific cytokines to assist in tissue regeneration/repair after lung injury. Recent analyses showed that tissue Mϕs constantly evolve their phenotype in response to their dynamic micro-environment during disease states. In contrast, under typical *in vitro* conditions, Mϕs exhibit either M1 or M2 phenotypic characteristics of activation, when cultivated with specific immunomodulatory Th1 or Th2 cytokines [5].

The phenotypic activation of Mϕs is also modified by intrinsic

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autocrine signals [6,7]. Resident Mϕs in several tissues reportedly produce γ-aminobutyric acid (GABA) from glutamate via glutamic acid decarboxylase (GAD) and express A-type GABA receptors ($GABA_AR$) [8–11]. This suggests that it is feasible to regulate Mϕs by an autocrine GABA signaling mechanism. Indeed, a recent study by Sanders *et al.* reported that activating $GABA_ARs$ using benzodiazepine inhibits AMϕ's phagocytic activity in *Streptococcus pneumoniae*-infected mouse lungs, resulting in increased mortality of mice due to worsened pneumonia [12]. On the other hand, we previously showed that airway and alveolar epithelial cells increase GABA secretion in the lung of mice experiencing an asthmatic reaction [13], a typical Th2/M2 pulmonary condition. Taken together, all the available data suggest that pulmonary environments may influence GABA signaling in LMϕs and lung epithelial cells, which in turn further regulates the pulmonary immune responses. Despite these findings, the cellular and molecular mechanisms by which auto- and paracrine GABA regulates the phenotypical activation of LMϕs remain to be determined. This study sought to explore whether autocrine GABA signaling in LMϕ adjusts to $M1/T_H1$ and $M2/T_H2$ environments, and, if so, whether it contributes to the LMϕs' phenotypic activation state under *in vivo* and *in vitro* conditions.

2. Methods

2.1. Animals and treatments

C57BL/6 mice (8–10 weeks old, Charles River Laboratories, Senneville, QC, Canada), BALB/c mice (8–10 weeks, Harlan, Indianapolis, IN, USA) and *lys*-EGFP-*ki* transgenic mice [14] (totally 106 mice) were used in the present study. The *lys*-EGFP-*ki* transgenic mice were originally obtained from Dr. Thomas Graf and bred by homozygous mating in the University of Western Ontario barrier facility. All experimental procedures conform to the Canadian Council on Animal Care guidelines for the care and handling of animals and the institutional animal research committee at Western University approved all studies (Animal Protocol Numbers: # 2007-104 and 2016-019, # 2010-038 and # 2010-272).

The *lys*-EGFP-*ki* mice are on a C57BL/6 genetic background and express enhanced green fluorescent protein (EGFP) in mature circulating and tissue myelomonocytic/granulocytic cells [14], which can be identified by fluorescence microscopy. To study the effect of systemic inflammation on the function of LMϕs, a low dose (1.0 mg/kg body weight, n = 6 mice) or a high dose (6.0 mg/kg body weight, n = 9 mice) of lipopolysaccharide (LPS [type *E. coli* 0111:B4, L4391 Sigma-Aldrich, St. Louis, MO, USA] in 100 μL phosphate-buffered saline [PBS]) was intravenously (i.v.) administered through the tail vein under anesthesia with isoflurane. Another 6 mice were injected with 6.0 mg/kg detoxified LPS (L3023, Sigma-Aldrich) in 100 μL PBS as control. In another two sets of experiments, an effective and safe dose of the selective $GABA_AR$ agonist muscimol (0.2 mg/kg) [15] and the $GABA_AR$ antagonist picrotoxin (0.5 mg/kg) [16] was i.v. administered respectively to mice (n = 5, each group), 30 min after injection with a high dose of LPS. Twenty four hours after LPS injections, all test *lys*-EGFP-*ki* mice were anesthetized by intraperitoneal (i.p.) ketamine-xylazine (85 mg/kg and 15 mg/kg, respectively, in 100 μL PBS), and then perfused transcardially with PBS followed by 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO, USA).

To study the role of $GABA_AR$ signaling in regulating the function of LMϕs under normal and inflammatory conditions, PBS (50 μL), the $GABA_AR$ agonist muscimol (25 μg in 50 μL PBS), the $GABA_AR$ antagonist picrotoxin (25 μg in 50 μL PBS) or a low dose of LPS (0.02 mg in 40 μL PBS) was intranasally (i.n.) administered into the lungs of naïve C57BL/6 mice (10–12 weeks old, 22–28 g) under ketamine-xylazine anesthesia. Eighteen hours after treatment, mice (n = 6 in each group) were euthanized by deep anesthesia using isoflurane and lungs were subjected to a bronchoalveolar lavage (BAL) with PBS that yielded 2 mL for cytokine- and cell-based assays (see below).

To study the effect of pulmonary Th2-type immune responses on the function of LMϕs, female BALB/c mice (n = 8) housed in environmentally controlled specific pathogen-free conditions were sensitized and challenged to develop allergic airway responses, as we previously described [17]. Briefly, under isoflurane-induced anaesthesia each mouse experienced an i.p. injection of ovalbumin (OVA, 10 μg in 200 μL PBS) on days 1 and 11. Also on day 11, these mice were given 100 μg of OVA in 25 μL PBS via the i.n. route. On day 30, all OVA-sensitized mice were challenged with 100 μg of OVA in 25 μL PBS via the i.n. route. Control mice (n = 8) were administered with 200 μL and 25 μL PBS via the i.p. and i.n. routes respectively. Subsequently, all mice were terminally anesthetized by i.p. injection of ketamine-xylazine and then perfused with PBS followed by 4% PFA on Day 30/31.

2.2. Lung tissue immunohistochemistry

The lungs were dissected and post-fixed in 4% PFA for 24 h. The tissues were then either embedded in paraffin, or cryoprotected successively in 10% and 30% sucrose for another 24 h before embedding in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). Lung sections were immunostained using our procedures described previously [17,18]. Briefly, lung sections were blocked in 10% normal serum for 2 h. After gentle rinses using PBS, lung sections were incubated overnight using the following primary antibodies: rabbit anti-GAD65/67 (1:500, G5163, Sigma-Aldrich), polyclonal rabbit anti-α2$GABA_AR$ (1:200, AGA-002, Alomone Labs, Jerusalem, Israel), anti-F4-80 (1:50, #CL8940AP, Cedarlane Labs, Burlington, NC, USA). Notably, F4-80, a transmembrane protein specifically present on the surface of mouse Mϕs [19], was used as a marker of Mϕs. The specificity of anti-α2$GABA_AR$ (AGA-002, Alomone Labs) for immunocytochemistry was previously substantiated in neuroprogenitor cells, which was validated by quantitative PCR analyses [20]. Lung sections were rinsed using PBS and then incubated with Cy3 or Alexa fluor 647-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 h. To ensure stain specificity, omission of primary antibodies was used as negative controls. The FITC-conjugated pan-anti-cytokeratin (1:200, #F3418, Sigma-Aldrich) was used to visualize lung epithelial cells.

2.3. Analyses of BAL cytokines and BAL cells

Naïve and LPS-treated C57BL/6 mice were anaesthetized using isoflurane (Baxter Corporation, Missisauga, ON, Canada) and then euthanized by cervical dislocation. An endotracheal tube (Becton Dickinson, Oakville, ON, Canada) was quickly inserted into the trachea. As reported previously [21], 1.0 mL Hanks balanced salt solution (HBSS, Life Technologies, Carlsbad, CA, USA) was instilled and withdrawn from the lung three times through the endotracheal tube, and the total volume (~2 mL) of BAL fluid was recorded. The lavage was centrifuged for 10 min at 400 × *g* at 4 °C for isolation of BAL cells. The lavage supernatant from this spin was snap frozen in liquid nitrogen for cytokine analysis. The pellet of BAL cells was resuspended in 200 μL of Dulbecco's PBS or Plasmalyte. Fifty μL of resuspended BAL cells were cytospun (Shandon Elliott Cytospin, John's Scientific, Toronto, ON, Canada) onto a slide and stained by HemaColor (EM Science, Gibbstown, NJ, USA) for differential cell analysis. In addition, the resuspended solution was combined with an equal volume of 0.4% trypan blue stain (Gibco by Thermo Fisher Scientific, Waltham, MA, USA), and total live cells were counted using a hemocytometer and light microscope. Macrophages in the BAL were identify by their unique morphology including their relative large size (25–50 μm in diameter) eccentrically placed nucleus and cytoplasmic vacuoles.

2.4. LMϕ isolation, culture and treatments

Our initial immunohistochemical assays revealed that $GABA_AR$-