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A novel role for RGMa in modulation of bone marrow-derived dendritic cells maturation induced by lipopolysaccharide

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ABSTRACT

Repulsive guidance molecule a (RGMa) is known to mediate immune responses and has been indicated to modulates T cell activation and autoimmune diseases by dendritic cells (DCs), which hints its significant function in the latter cells. The aim of our study, therefore, was to evaluate the function of RGMa in DC maturation. We found that small interfering RNA (siRNA) successfully silenced the expression of RGMa in DCs. Even after LPS stimulation, RGMa-silenced DCs displayed an immature morphology, characterized by small, round cells with a few cell processes and organelles, and many pinocytotic vesicles. In the presence of LPS, RGMa siRNA transfection markedly reduced levels of CD80, CD86, CD40, and MHC II expression, as well as the secretion of IL-12p70 and TNF-α. With LPS treatment, RGMa siRNA-transfected DCs also showed increased levels of IL-10 and endocytosis. Moreover, in the presence of LPS, RGMa siRNA-transfected DCs displayed a low ability to induce T cell proliferation and differentiation, compared with negative control (NTi)-transfected or control DCs ($p < 0.05$ for both). We conclude that after LPS stimulation, RGMa siRNA-transfected DCs show immunoregulatory and tolerogenic characteristics, which provides new insights into the immune system.

1. Introduction

As professional antigen-presenting cells (APCs), dendritic cells (DCs) harbor an extraordinary capacity to stimulate naive T cells, generate primary and secondary immune responses against foreign antigens, and serve as a bridge connecting the innate and adaptive immune systems [1, 2]. Under normal physiological conditions, DCs remain immature in almost all peripheral tissues, and are characterized by a high phagocytic capacity, but have poor abilities for antigen presentation and costimulatory molecule expression [3, 4]. After encountering pathogens, DCs initiate a maturation process that results in the enhanced expression of MHC and co-stimulatory molecules, and in high levels of certain cytokines, which subsequently activate naive T cells to induce Th1 or Th2 polarization [5, 6]. A number of studies have confirmed that differing mature states of DCs can influence the immune response [7, 8]. Moreover, DCs not only modulate the immune response, but are also involved in the immune tolerance; such cells are called tolerogenic dendritic cells (tolDCs) [9, 10]. TolDCs have the function of immune regulation, and may offer an intervention therapy in autoimmune diseases.

Anchored to the cell membrane by covalent linkage to glycosylphosphatidylinositol, the repulsive guidance molecule (RGM) family includes three members, RGMa, RGMb, and RGMc. RGM molecules are restrictively expressed in a small number of tissues and are considered to have distinct biological functions, including iron metabolism regulation, neuronal survival, cell adhesion, and endochondral bone formation [11–13]. More recently, researchers have shown that RGMa can also be expressed in spleen, monocytes, and lymphocytes, which implicates RGMa in the immune response [14]. Moreover, a study on multiple sclerosis (MS) and a mouse model of myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), in which DCs play an irreplaceable role, has demonstrated that RGMa can modulate the clinical severity of EAE by DCs [15]. It is also found that the expression of RGMa in DCs increases after the induction of mouse EAE, and the mature DCs obtained from autopsied MS samples are immunoreactive for RGMa. These studies highlight the notion that RGMa can control DC maturation, and may play a significant role in the immune tolerance and regulation. Despite this, conclusive evidence of a role for RGMa in DC function is lacking, underlining the pressing need for further investigation of this molecule

Abbreviations: APCs, antigen presenting cells; CNS, central nervous system; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage–colony-stimulating factor; IRAK-4, interleukin-1 receptor-associated kinase 4; IRF- 3, interferon regulatory factor 3; LPS, lipopolysaccharide; MACS, magnetic activated cell sorting; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NTi, negative control; PBS, phosphate balanced solution; RBC, red blood cells; RGMa, repulsive guidance molecule a; SEM, scanning electron microscopy; ShcA, Src homology 2 domain-containing A; siRNA, small interfering RNA; SOCS3, suppressor of cytokine signaling 3; TEM, transmission electron microscopy; tolDCs, tolerogenic dendritic cells.

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in such cells. Therefore we conducted the following investigation to look at concrete happening of this.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice, 4–6 weeks of age, were obtained from the Medical Experimental Animal Center, China Medical University, Shenyang, China. All mice were kept under specific pathogen-free conditions, with water and food provided ad libitum.

2.2. Key chemicals

Recombinant GM-CSF and IL-4 were purchased from PeproTech Inc. (Rock Hill, NJ, USA). RGMa and NTi siRNA were bought from Qiagen (Hilden, Germany). A Naive T Cell $CD4^+/CD62L^+/CD44^{low}$ Column Kit was obtained from R&D Systems Inc. (Minneapolis, MN, USA). Anti-RGMa antibody was purchased from Abcam PLC (Cambridge, UK), and anti-NF-κb p65 antibody was purchased from Cell Signaling Technology Inc (Beverly, MA, USA). The PrimeScript™ RT reagent and SYBR® Premix Ex Taq™ II kits were from Takara (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits and mAbs in flow cytometry were all from eBioscience Inc. (San Diego, CA, USA). The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit was purchased from Promega (Madison, WI, USA). The remaining chemicals were purchased from Sigma Aldrich or BD Pharmingen (San Jose, CA, USA).

2.3. Preparation and purification of bone marrow-derived DCs in culture

The protocol described by Inaba et al. [16] was used to prepare DCs in our study. After sacrificing mice, marrow cells were extracted using a syringe to flush the bones, and any remaining red blood cells (RBCs) were lysed with RBC lysing buffer. Afterwards, the remaining cells were placed in bone-marrow derived dendritic cell (BMDC) medium (RPMI 1640 with 10 ng/ml recombinant GM-CSF and IL-4). On day 3, the old medium was replaced with fresh BMDC medium. Half the medium was changed every other day. After 5 days, non-adherent and non-proliferating aggregates of immature DCs were harvested and CD11c positive selection kits and magnetic activated cell sorting (MACS) columns (Miltenyi Biotec, Auburn, CA, USA) were used to purify DCs for further studies. DC purity was evaluated by flow cytometric analysis, with a target purity of >95% (data not shown). To induce DC maturation, 1 μg/mL LPS was added to the culture on day 6 after transfection, and cells were collected after 24 h stimulation.

2.4. Isolation of T lymphocytes

T lymphocytes were purified from spleen cells isolated from C57BL/6 mice using a Mouse Naive T Cell $CD4^+/CD62L^+/CD44^{low}$ Column Kit. Methods used were strictly in accordance with the kit's instructions. In brief, pre-washed, pre-packaged enrichment columns were prepared and washed with column buffer. After depleting RBCs performed as above, spleen cells were suspended in column buffer. Cell suspensions were then mixed with a monoclonal antibody cocktail, incubated at room temperature for 15 min, and columns were used to isolate purified T lymphocytes. Isolated cells were 90–95% CD4-positive T lymphocytes.

2.5. RNA silencing

For transient transfection experiments, immature DCs were harvested as outlined above. Before transfection, cells (2×10^5) were seeded in 100 μL of BMDC medium. Transfection complexes of RGMa or NTi siRNA and Effectene Transfection Reagent were then added to the culture medium, and cells were incubated under normal growth conditions. After 6 h of incubation, 900 μL BMDC medium was added and cells were further incubated until analyzed for gene silencing. Transfection efficiency

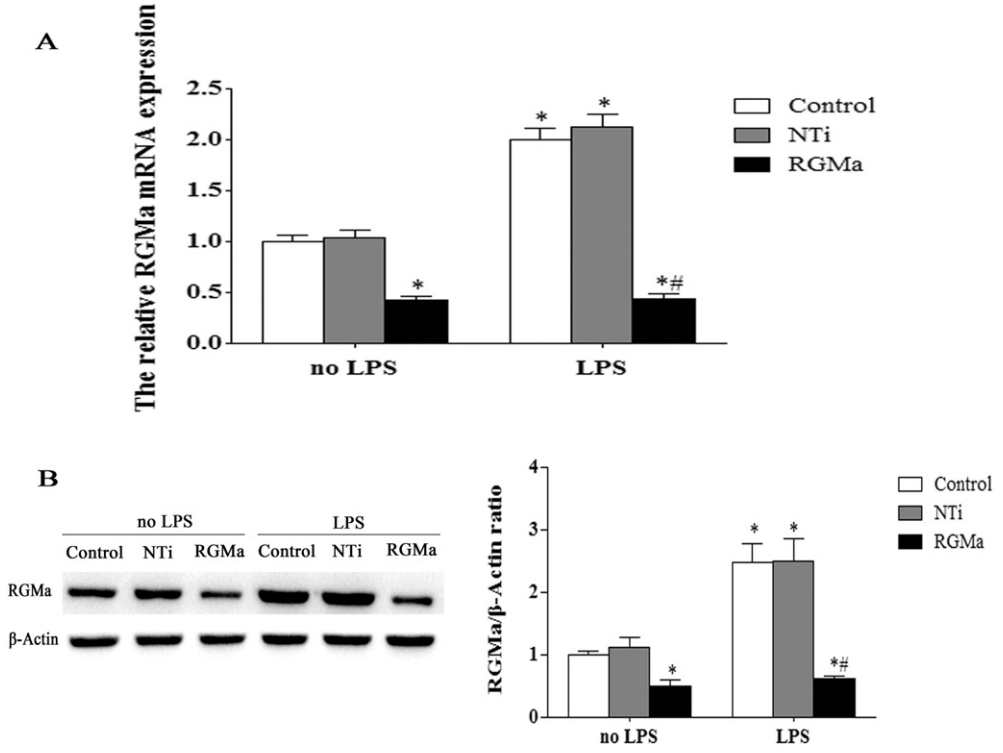

Fig. 1. The interference efficiency of RGMa siRNA in DCs. (A) Real-time PCR indicated the relative RGMa mRNA expression after RGMa or NTi siRNA was transfected into DCs, with or without 1 μg/mL LPS stimulation. (B) Western blotting showed the relative expression level of RGMa/β-actin in DCs after transfection. Band intensities indicated the protein expression of RGMa, and β-actin was used as a protein loading control. Data were expressed as mean ± S.E.M. of three independent experiments. $^*p < 0.05$ vs. control DCs without LPS treatment. $^\#p < 0.05$ vs. control or NTi siRNA-transfected DCs with LPS treatment.