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Liposomes of phosphatidylcholine and cholesterol induce an M2-like macrophage phenotype reprogrammable to M1 pattern with the involvement of B-1 cells

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ABSTRACT

Macrophages respond to endogenous and non-self stimuli acquiring the M1 or M2 phenotypes, corresponding to classical or alternative activation, respectively. The role of B-1 cells in the regulation of macrophage polarization through the secretion of interleukin (IL)-10 has been demonstrated. However, the influence of B-1 cells on macrophage phenotype induction by an immunogen that suppress their ability to secrete IL-10 has not been explored. Here, we studied the peritoneal macrophage pattern induced by liposomes comprised of dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol) carrying ovalbumin (OVA) (Lp DPPC/OVA), and the involvement of B-1 cells in macrophage polarization. Peritoneal cells from BALB/c, B-1 cells-deficient BALB/*xid* and C57BL/6 mice immunized with Lp DPPC/OVA and OVA in soluble form (PBS/OVA) were analyzed and stimulated or not *in vitro* with lipopolysaccharide (LPS). Peritoneal macrophages from BALB/c and C57BL/6 mice immunized with Lp DPPC/OVA showed an M2-like phenotype as evidenced by their high arginase activity without LPS stimulation. Upon stimulation, these macrophages were reprogrammable toward the M1 phenotype with the upregulation of nitric oxide (NO) and a decrease in IL-10 secretion. In addition, high IFN-γ levels were detected in the culture supernatant of peritoneal cells from BALB/c and C57BL/6 mice immunized with Lp DPPC/OVA. Nevertheless, still high levels of arginase activity and undetectable levels of IL-12 were found, indicating that the switch to a classical activation state was not complete. In the peritoneal cells from liposomes-immunized BALB/*xid* mice, levels of arginase activity, NO, and IL-6 were below those from wild type animals, but the last two products were restored upon adoptive transfer of B-1 cells, together with an increase in IFN-γ secretion. Summarizing, we have demonstrated that Lp DPPC/OVA induce an M2-like pattern in peritoneal macrophages reprogrammable to M1 phenotype after LPS stimulation, with the involvement of B-1 cells.

Abbreviations: APCs, antigen presenting cells; APC, allophycocyanin; Chol, cholesterol; PE Cy7, cyanine dye (Cy7) combined with phycoerythrin; CS, culture supernatant; DPPC, dipalmitoylphosphatidylcholine; Lp DPPC/OVA, DPPC and Chol liposomes encapsulating OVA; FITC, fluorescein isothiocyanate; FSC-A, forward scatter-area; GM-CSF, granulocyte-macrophage colony-stimulating factor; α-Gal Cer, α-galactosyl-ceramide; iNOS, inducible NO synthase; IL, interleukin; LPM, large peritoneal macrophage; LXRs, liver X receptors; M-CSF, macrophage colony-stimulating factor; MFI, median fluorescence intensity; NK, natural killer; NO, nitric oxide; Non-Imm, non-immunized; ND, not detected; OVA, ovalbumin; PB, Pacific Blue; PerC, peritoneal cavity; PE, phycoerythrin; SSC-A, side scatter-area; SPM, small peritoneal macrophages; Th1, T helper 1; Th2, T helper 2.

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Introduction

Macrophages are immune cells that play a critical role in sensing pathogens to regulate the adaptive immune response. On the basis of the T helper 1 (Th1)/T helper 2 (Th2) polarization concept (Romagnani 2000), functional phenotypes of macrophages are generally termed pro-inflammatory M1 or classically activated, and anti-inflammatory M2 or alternatively activated (Gordon 2003; Martinez et al. 2009).

Macrophages are polarized to the M1 phenotype by exposure to Th1 cytokines such as IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), or in the presence of bacterial products such as LPS. M1 macrophages are characterized by high IL-12 and IL-23, and low expression of the anti-inflammatory cytokine IL-10, by the production of reactive nitrogen and oxygen intermediates and pro-inflammatory cytokines, such as IL-6 and TNF-α, and by the upregulation of molecules associated with antigen presentation, as MHC class II and costimulatory molecules CD40, CD80, and CD86. M1 macrophages are microbicidal and generally promote Th1 and Th17 responses (Benoit et al. 2008; Khallou-Laschet et al. 2010; Mantovani and Locati 2009). M2 macrophages are polarized by stimulation with Th2 cytokines, such as IL-4 and IL-13, as well as macrophage colony-stimulating factor (M-CSF) (Gordon and Martinez 2010). They have upregulated expression of arginase, scavenger and mannose receptors, FIZZ1, the IL-1R antagonist, and the chitinase family protein Ym1 and also express low levels of IL-12 and high levels of IL-10. M2 macrophages have efficient phagocytic activity, but are usually ineffective at killing microbial pathogens, and are associated with anti-inflammatory and Th2-type responses, wound healing, and resolution of inflammation (Benoit et al. 2008; Mantovani et al. 2004a,b). However, *in vivo*, the division between M1 and M2 cells may be blurred, with the above phenotypes likely representing two extremes in a continuum of macrophage functional states (Mantovani et al. 2004a,b; Mantovani et al. 2002).

The mouse peritoneal cavity (PerC) is a compartment in which a variety of immune cell populations reside, being the macrophages one of the most abundant. Two macrophage subsets, known as small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM), with different phenotypic, functional, and developmental characteristics, have been identified in the PerC. Both SPM and LPM express the typical F4/80 and CD11b macrophage surface markers and show clear phagocytic activity *in vivo*. However, they differ sharply in the expression of several surface molecules, including Gr-1 and MHC-II, and show strikingly different response patterns after activation with typical macrophage stimuli, such as LPS or thioglycolate (Ghosn et al. 2010).

Another cell population with high frequency in the PerC is the B-1 cells, a unique subset of B cells involved in innate immunity, autoimmunity, and immune regulation. These cells are the primary producers of natural IgM, providing the first line of protection against various virus and bacteria (Baumgarth et al. 2000a,b; Berland and Wortis 2002). B-1 cells are also a major source of IL-10 (O'Garra et al. 1992). It was previously demonstrated that B-1 cells influence the effector functions of macrophages by decreasing phagocytic indexes, and nitric oxide (NO) and hydrogen peroxide production (Barbeiro et al. 2011; Popi et al. 2004). B-1 cells were also shown to polarize macrophages to an M2-like phenotype both *in vitro* and *in vivo* (Wong et al. 2010). In these effects, B-1 cells-derived IL-10 had a crucial role.

Liposomes are lipid vesicles frequently used as adjuvants due to their capacity to encapsulate and transport several types of antigens and to potentiate the antigen-specific immune response (Watson et al. 2012). The ability of liposomes to interact with different immune cells, such as dendritic cells and macrophages, has been well documented (Steinman 2008; Thiele et al. 2001). We recently confirmed that liposomes comprised of DPPC and Chol and encapsulating OVA (Lp DPPC/OVA), enhance the antigen-specific humoral response exhibiting a mixture Th1/Th2 pattern and demonstrated the activation of mouse peritoneal B-1 cells by this preparation (Cruz-Leal, submitted). The involvement of B-1 cells in the anti-OVA response was mediated by the cells themselves and probably the antibodies secreted by them. In addition, the liposomal preparation induced an increase in anti-phosphocholine IgM production, B-1 cell differentiation into phagocytes (Cruz-Leal, submitted), and a reduction in IL-10 secretion (Cruz-Leal, unpublished data). Taking into account these previous findings, the aim of the present study was to determine the pattern of peritoneal macrophages induced by Lp DPPC/OVA in mouse models, and the participation of B-1 cells in macrophage polarization. Herein, we show that Lp DPPC/OVA provoked an increase in the SPM subpopulation. Also, this immunogen led to an increase in MHC-II expression by the LPM population. Peritoneal macrophages from BALB/c and C57BL/6 mice immunized with Lp DPPC/OVA displayed an M2-like phenotype as evidenced by high arginase activity, without *in vitro* stimulation. Interestingly, these macrophages could be reprogrammed to an M1 pattern after stimulation with LPS, as demonstrated by the production of high levels of NO and low levels of IL-10. Moreover, the culture supernatant of peritoneal cells from BALB/c and C57BL/6 mice immunized with Lp DPPC/OVA showed high IFN-γ levels. The polarization to a classical activation state was however partial, as shown by the high levels of arginase activity and undetectable IL-12 secretion after LPS stimulation. In addition, we demonstrated that in B-1 cells-deficient BALB/*xid* mice the liposomal preparation was not able to induce the levels of arginase activity and NO secretion detected in macrophages from BALB/c mice. The involvement of B-1 cells in this phenomenon was proven after adoptive transfer of B-1 cells to BALB/*xid* mice. When reconstituted animals were immunized with Lp DPPC/OVA, their peritoneal cells did not show an increase in arginase activity, but the levels of NO and IL-6 upon stimulation with LPS behaved as those from immunized BALB/c mice, and interestingly the IFN-γ levels were even higher.

Materials and methods

Reagents

OVA grade V used as model antigen in immunization protocols and DPPC and Chol used for generation of liposomes were purchased from Sigma–Aldrich (St. Louis, MO) and Northern Lipids (Vancouver, Canada), respectively.

Mice

Male BALB/c, BALB/*xid* and C57BL/6 mice, 6–10 week old, were purchased from the Development Center of Experimental Models for Medicine and Biology of the Federal University of São Paulo, Brazil. All animals were specific pathogen-free and maintained under standard animal house conditions with free access to water and standard rodent pellets. Experiments were performed in accordance with institutional guidelines.

Encapsulation of OVA into liposomes and characterization of vesicles

The procedure based on dehydration and rehydration of vesicles developed by Kirby and Gregoriadis (1984) was used to obtain liposomes encapsulating OVA. Briefly, small unilamellar vesicles comprised of DPPC and an equimolar quantity of Chol were generated by ultrasonication and then mixed with OVA. After freezing