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Pertussis toxin-induced cytokine differentiation and clonal expansion of T cells is mediated predominantly via costimulation ☆

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Abstract

Pertussis toxin (PTX) has potent immunologic adjuvant activity *in vivo* and concomitantly enhances both T helper type (Th1) and Th2 cytokine responses. The PTX-induced enhancement of Th1 and Th2 immunity is mediated via the activation of antigen presenting cells (APCs), but the underlying mechanism is not known. Here we asked whether the adjuvant activity of PTX on T cell immunity was mediated by cytokines and/or costimulatory signals. The results show that *in vivo* blockade of CD28–CD80/86 costimulation essentially abrogated PTX-mediated enhancement of antigen-specific Th1 and Th2 responses. Blockade of CD40L–CD40 interactions was less efficient in inhibiting PTX-mediated enhancement of Th1 and Th2 responses. In contrast, the adjuvant activity of PTX was not mediated via cytokines, because neither Th1 nor Th2 responses were substantially impaired in mice deficient for IL-12, IFN-γ, IL-4, IL-5, or IL-6. Collectively, the data suggest that PTX mediates its adjuvant effects on T cell cytokine differentiation and clonal expansion via the modulation of costimulatory molecules on APCs. Understanding the costimulatory pathways targeted by PTX could lead to the design of novel adjuvants that selectively induce Th1 or Th2 immunity.

Keywords: Pertussis toxin; T cell expansion; Cytokines; Costimulatory molecules; Adjuvant; T cell; Costimulation

1. Introduction

T cells can be classified into subpopulations based on their functional properties and cytokine profiles. T helper type 1 (Th1) cells are characterized by the production of IFN-γ and IL-2, whereas T helper type 2 (Th2) cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 [1].

However, in order to produce these cytokines, T cells have to be activated by the recognition of cognate antigen on APCs and receive signals through costimulatory molecules. APCs are thought to modulate the cytokine differentiation of T cells via the secretion of IL-12 to induce Th1 cells, or IL-4 and IL-6 to promote Th2 responses [2,3]. Interestingly, immunologic adjuvants, such as complete Freunds' adjuvant (CFA[1]; mineral oil containing inactivated mycobacteria), activate APCs and modulate the cytokine differentiation of the ensuing T cell response. For example, injection of protein antigens emulsified in

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[1] *Abbreviations used:* PTX, pertussis toxin; Th, T helper; IL, interleukin; Ig, immunoglobulin; IFN, interferon; HEL, hen eggwhite lysozyme; OVA, ovalbumin; IFA, incomplete Freund's adjuvants; CFA, complete Freund's adjuvants; KO, knockout; mAb, monoclonal antibodies; Alum, Aluminum hydroxide; APCs, antigen presenting cells.

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CFA induces polarized type 1 immunity as defined by the production of IFN-γ and IL-2, but not IL-5, and IgG2a antibodies [4]. In contrast, injection of the same antigens in incomplete Freunds' adjuvant (IFA; mineral oil in the absence of microbial products) results in type 2 immune responses as defined by the production of IL-4, IL-5, IgG1 and IgE, but not IFN-γ [5]. This information is useful, because the modulation of the immune response is critical for certain infectious diseases, such as leprosy or leishmanosis, in which the balance of Th1 to Th2 cells defines the outcome of the disease. So far only Alum and IFA, both type-2 adjuvants, are approved for human use, and there is a great surge of interest in developing adjuvants that selectively induce type-1 immunity.

Recent studies have shown that other microbial products, such as CpGs, *Pertussis* toxin (PTX) or *Cholera* toxin, also have adjuvant effects [6–8]. Interestingly, PTX has been used for many years to enhance the induction of organ-specific autoimmune diseases elicited by immunization of laboratory animals with the appropriate tissue autoantigens [8,9].

The mechanism by which PTX promotes immune responses is not fully understood. It is known that PTX is taken up into cells and leads to irreversible ADP ribosylation of the Gi-subclass of G proteins [10]. Recent studies have suggested that PTX may act through the activation of TOLL-like receptors (TLRs) [11]. Depending on the adjuvant used for immunization, i.e. CFA or IFA, PTX has the interesting property of simultaneously promoting clonal expansion of Th1 and Th2 cells to co-injected antigens [12,13]. The PTX-mediated, enhanced antigen-specific cytokine production originates from clonally expanded Th1 and Th2 cells, but not from Th0 cells [12]. T cell expansion seems to be mediated via the activation of APCs, as indicated by an increased expression of MHC class II molecules, costimulatory molecules, and cytokine production by APCs following exposure to PTX. Subsequently, PTX-activated APCs may result in enhanced differentiation and clonal expansion of Th1 and Th2 cells in lymphoid tissues [8,13].

In the present study, we asked whether the adjuvant activity of PTX was dependent upon cytokines and/or signaling via costimulatory molecules.

The results show that the adjuvant effect of PTX is primarily dependent on co-stimulatory molecules. CD80 and CD86 interactions with CD28 were most important for the enhancement of both Th1 and Th2 subset of T cells, while the CD40/CD40L pathway played a lesser role. In contrast, no single cytokine tested was obligatory for PTX-mediated adjuvant effects.

2. Materials and methods

2.1. Animals, antigens, and treatments

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at Case Western Reserve University (CWRU) under specific pathogen free conditions. IL12p35-deficient, IL-$5^{tm1Kopf}$-deficient, IL-4^{tm1Nnt}-deficient and IL-$6^{tm1Kopf}$-deficient on a C57BL/6-background were used as knockout-models (Jackson Laboratories, Bar Harbor, ME). All animal procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of CWRU. Female BALB/C and C57BL/6 mice were injected at 6–10 weeks of age with the antigens (see below) in IFA or Alum. Pertussis toxin (PTX, 200 ng, List Biological Laboratories, Campbell, CA) was injected intraperitoneally in 500 μl saline at 0 and 24 h after injection of the test antigen as indicated in the text. IFA was purchased from Gibco BRL, Grand Island, NY, and Alum from Pierce Chemical Company (Rockford, IL). Hen eggwhite lysozyme (HEL) and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). HEL106-116 peptide was synthesized by Princeton Biomolecules Corporation (Langhorne, PA). Antigens were mixed with the adjuvants to yield a 1 mg/ml emulsion, of which 100 μl was injected subcutaneously. Blocking anti-CD80- (16-10A1), anti-CD86- (GL1) and CD40L mAb (MR-1) were gifts of Dr. Frederick Heinzel (Case Western Reserve University, Cleveland, OH). The mAb (0.5 mg/mouse) were injected intraperitoneally in 0.5 ml of saline as indicated in the figure legends.

2.2. Cell preparations from the organs tested

Single cell suspensions from the spleen, lymph node or peritoneal lavage were prepared as described previously [8,12]. The cells were counted and plated with antigen in HL-1 serum-free medium (BioWhittaker, Walkersville, MD) at 1×10^6 cells per well, and tested as indicated in the text.

2.3. Cell separations

Single cell suspensions were prepared from spleens. $CD4^+$ T cells were obtained by passing the spleen cells through a murine $CD4^+$ T cell enrichment column (R&D Systems, Minneapolis, MN), following the manufacturer's suggested protocol. Flow cytometry analysis (FACScan, BD Biosciences) showed more than 95% enrichment for $CD4^+$ cells. Irradiated APCs from naïve BALB/c mice were added at 1×10^5 cells per well as indicated in Fig. 4.

2.4. Cytokine measurements by ELISPOT and computer-assisted ELISPOT image analysis

The ELISPOT assay was performed as described previously [12]. ELISPOT plates (Multiscreen IP, Millipore) were coated overnight with specific capture antibody (IFN-γ, AN-18, 2 μg/ml; IL-2, JES6-1A12, 4 μg/ml; IL-5, TRFK5, 4 μg/ml; all eBioscience; IL-4, 11B11, 4 μg/ml; BD Pharmingen) diluted in 1× PBS. The plates were blocked with 1% BSA in PBS, for 1 h at room temperature, and then washed 4 times with PBS. Spleen cells were plated