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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 (Th) 1 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

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

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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 Freund's adjuvant (CFA; 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 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 Freund's 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 leishmaniasis, 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 μ l saline at 0 and 24 hours 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 μ l 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 μ g/ml; IL-2, JES6-1A12, 4 μ g/ml; IL-5, TRFK5, 4 μ g/ml; all eBioscience; IL-4, 11B11, 4 μ g/ml; BD Pharmingen) diluted in $1 \times$ 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 at 1×10^6 cells/well, alone or with antigen (7 μ M) in HL-1 serum free medium and cultured for 24 h for IFN- γ , or IL-2, or 48 h for IL-4 or IL-5. Subsequently, the cells were removed by washing with $4 \times$ PBS and $4 \times$ PBS/Tween, and the respective biotinylated detection antibody (IFN- γ , R4-6A2, 2 μ g/ml; IL-2, JES6-5H4, 2 μ g/ml; IL-4, BVD6-24G2, 2 μ g/ml; IL-5, TRFK4; all eBioscience) was added and incubated overnight. The plate-bound secondary antibody was then visualized by adding streptavidin-alkaline phosphatase (SAV-AP, Dako Carpinteria, CA) and NBT/BCIP substrate (Biorad, Hercules, CA/Sigma, St. Louis, MO). Image analysis of ELISPOT assays was performed on a Series 2 ImmunoSpotTM Image Analyzer (Cellular Technologies, Cleveland, OH). Digitized images of individual wells of the ELISPOT plates were analyzed for cytokine spots, based on the comparison of experimental (containing T cells and antigen presenting cells with antigen) and control wells (T cells and antigen presenting cells, no antigen). After separation of spots that touched or partially overlapped, non-specific noise was gated out by applying spot size and circularity analysis as additional criteria. Spots that fell within the accepted criteria were highlighted and counted. The stimulation index was calculated by dividing the number of cytokine spots detected in wells pulsed with cognate antigen by the number of cytokine spots in wells without antigen (medium only). The spot number in unimmunized or control mice (irrelevant antigen) was in the same range as the medium controls.

2.5 Measurement of specific serum antibodies

The assay was performed as described previously [4,14]. In brief, plates (Nunc Immunoplate, Fisher Scientific, Pittsburg, PA) were coated with HEL (10 μ g/ml) diluted in 0.1 M bicarbonate coating buffer overnight at 4°C, then blocked for 1–2h with 0.1% gelatin in PBST. The test serum was diluted 1:1500 then added and incubated overnight at 4°C. Biotinylated anti-mouse

IgG (H+L), IgG1, or IgG2a (Zymed) was added and refrigerated overnight. The following day, plate-bound antibody was detected by adding SAV-AP followed by PNPP for development of the colorimetric reaction. The relative OD units were measured with an ELISA reader.

3. Results

3.1 PTX-induced Th1 and Th2 differentiation is not primarily cytokine dependent

Pertussis toxin substantially enhances the T cell response to co-injected protein antigens [12, 13]. PTX increases the expression of MHC class II and costimulatory molecules on APCs, and enhances the release of innate cytokines, including IL-12 [12]. Hence, the PTX-mediated expansion of antigen-specific T cells could be driven by enhanced costimulation, and/or by cytokines released by innate immune cells.

To begin to address this issue, C57BL/6 WT mice were injected with OVA in IFA (OVA:IFA), with or without PTX. Additionally, groups of C57BL/6 cytokine knockout mice (deficient for IFN- γ , IL-4, IL-5, IL-6, or IL-12) were injected with OVA:IFA and PTX. Finally, one group of C57BL/6 WT mice was injected with OVA:IFA and PTX, and then treated with anti-IL-2 mAb. Cytokine profiles and frequencies of OVA-specific Th1 and Th2 cells were measured after 3 weeks by cytokine ELISPOT assay.

Confirming previous reports [12,13], C57BL/6 WT mice injected with OVA:IFA and PTX showed vigorous antigen-induced production of IFN- γ , IL-2, IL-4, and IL-5, consistent with the concomitant generation of Th1 and Th2 immunity (Fig. 1a–d, second bar from the left). The increase in IFN- γ , IL-2, IL-4, and IL-5 as compared with C57BL/6 WT mice injected with OVA:IFA alone (no PTX) was 20-, 10-, 2- and 10-fold respectively (Fig. 1a – d, first bar from the left). As expected, *in vivo* treatment with anti-IL-2 mAb abrogated cytokine production by all T cells (except for IL-4), consistent with its critical role for T cell proliferation. Importantly, vigorous PTX-induced Th1 and Th2 cytokine production was detected in knockout mice deficient in IL-4, IL-5, IL-6, IL-12, and IFN- γ , as compared with C57BL/6 WT mice (Fig. 1a – d). Specifically, the frequencies of IL-2 and IL-4 producing cells in the cytokine knockout mice injected with OVA:IFA and PTX were comparable to the frequencies observed in WT mice injected with OVA:IFA and PTX (Fig. 1b & c). Furthermore, production of IL-5 was not altered in mice deficient in IFN- γ , IL-6, and IL-12, but an approximately 50% reduction in IL-5 producing cells was observed in IL-4 knockout mice ($p < 0.05$). Finally, vigorous OVA-specific production of IFN- γ was detected in OVA:IFA and PTX injected mice deficient for IL-4, IL-5, IL-6, and IL-12. However, as compared with the WT mice, the frequencies of IFN- γ producing cells were significantly reduced in IL-4, IL-6, and IL-12 KO mice.

Collectively, the data show that PTX induced robust Th1 and Th2 cytokine production *in vivo*, independent of the presence of any particular cytokine tested in the animals, except for IL-2. The reduced numbers of T cells secreting particular cytokines in some mutant mice as compared with WT mice suggested an additive effect for certain cytokines for T cell expansion, such as IL-12 or IL-4. Lastly, in the absence of Th2 cytokines, PTX-induced Th1 differentiation was not increased, and vice versa (Fig. 1a–d).

3.2 CD80/CD86 blockade abrogates PTX-induced Th1 and Th2 differentiation in vivo

As shown, vigorous PTX-induced Th1 and Th2 cytokine production was detected in cytokine deficient mice. Thus, we asked whether PTX mediated Th1 and Th2 cytokine differentiation via enhanced costimulation by APCs. To address this issue, C57BL/6 mice were immunized with OVA:IFA and PTX. The mice were treated with anti-CD80 mAb, anti-CD86 mAb, anti-CD80 and anti-CD86 mAb combined, or anti-CD40L mAb, starting on day 2 after immunization, and weekly thereafter, for a total of three injections. Three weeks after the

immunization, the cytokine profiles and frequencies of OVA-reactive T cells were determined in single cell suspensions of spleen cells by cytokine ELISPOT assays.

The results show that treatment with either anti-CD80- or anti-CD86 mAb did not significantly affect the production of IFN- γ or IL-2 cytokines by Th1 cells (Fig. 2a & b). Similarly, treatment with anti-CD40L mAb did not significantly reduce the production of IFN- γ or IL-2 by OVA-reactive T cells. In contrast, treatment with either antibody alone decreased OVA-specific production of IL-5 by Th2 cells by 37.2 to 56.5% (Fig. 2c). Importantly, combined anti-CD80/CD86 mAb treatment essentially abrogated PTX-induced production of IFN- γ , IL-2, or IL-5 (Fig. 2a – c). Treatment with anti-CD40L mAb decreased the production of IFN- γ by OVA-reactive T cells (statistically not significant), but was basically without effect on the release of IL-2 (Fig. 2a, b). However, anti-CD40L mAb treatment significantly reduced the PTX-induced release of IL-5 in this model.

The requirement for costimulation for PTX-driven Th1 and Th2 cytokine production was not unique to C57BL/6 mice injected with OVA, since similar results were obtained in BALB/c mice immunized with HEL:Alum and PTX (Fig. 3). Specifically, combination anti-CD80/86 mAb treatment abrogated the production of IFN- γ , IL-2, and IL-5 (Fig. 3a – c). Treatment with anti-CD80 mAb alone did not significantly reduce cytokine production, whereas anti-CD86 treatment decreased production of IL-2 and IL-5, but not IFN- γ . Anti-CD40L treatment showed a tendency to decrease PTX-mediated production of all three cytokines (statistically significant for IL-2). Finally, similar results were obtained when BALB/c mice were immunized with HEL106-116 peptide in Alum and PTX (data not shown).

To directly show that costimulatory blockade decreased PTX-induced Th1/Th2 differentiation and expansion of T cells *in vivo*, CD4⁺ T cells were isolated from HEL:Alum and PTX injected BALB/c mice that were treated with anti-CD80/86 or anti-CD40L mAb, and tested in cytokine ELISPOT assays with naïve (untreated) APCs (Fig. 4). The data confirmed the earlier results and showed that cytokine production by T cells from anti-CD80/86 treated mice remained abrogated even in the presence of naïve (not with mAb) treated APCs. Anti-CD40L treated mice showed a decrease in IFN- γ and IL-2 producing T cells, whereas the production of IL-5 remained unaffected.

Collectively, the data show that the PTX-driven expansion of antigen-specific Th1 and Th2 cells is critically dependent on costimulatory signals, in particular via CD80/86 molecules.

3.3 CD80/86 costimulatory blockade inhibits Th1 and Th2 cytokine-mediated IgG subclass switching

The blockade of costimulation via CD80/86 and CD40L resulted in the inhibition of PTX-driven Th1 and Th2 cell expansion. To gain further insights into the biological relevance of this finding, we asked whether the costimulatory blockade affected antigen-specific antibody production in the animals.

In order to address this question, BALB/c mice were immunized with HEL:Alum and PTX and treated with anti-CD80- and anti-CD86 mAb combined, or with anti-CD40L mAb. Three weeks after immunization, serum antibodies were measured by ELISA assay as described in the material and methods section.

The results of the measurements of total serum immunoglobulin (Ig), IgG1, and IgG2a, are shown in Fig. 5. The data show that mice immunized with HEL:Alum and PTX produced high titers of total Ig, IgG1, and IgG2a antibodies, consistent with the mixed Th1/Th2 cytokine profile of the PTX-induced T cell response (Fig. 5a–c, open circles). However, inhibition of CD80/86 mediated costimulation essentially abrogated HEL-specific IgG1 and IgG2a antibody

production (Fig. 5a–c, closed triangles). This finding is consistent with the inhibition of PTX-mediated T cell responses by CD80/86 blockade (Figs. 2–4). In contrast, anti-CD40L mAb treatment preferentially inhibited the production of IgG2a antibodies (IFN- γ dependent), but had little effect on the production of IgG1 antibodies. This finding is in line with the results obtained earlier in BALB/c mice (Fig. 3 & 4), where anti-CD40L mAb treatment was also more efficient in inhibiting Th1 responses.

Taken together, the data show that PTX-mediated antibody production and IgG subclass switching are also costimulation dependent. The result provide a second line of evidence that costimulation is critical for PTX-mediated adjuvant effects. Our results suggest an important role for CD80/86, and to a more variable degree for CD40L, as mediators of the adjuvant effects of PTX.

4. Discussion

In this study, we show that the adjuvant activity of PTX on T cell cytokine differentiation and expansion is critically dependent on signals transmitted via costimulatory molecules, but not on signals mediated by cytokines. Inhibition of signaling via CD80 and CD86 abrogated PTX-driven Th1 and Th2 cell cytokine differentiation and expansion, and also prevented T cell-dependent antibody production. Inhibition of CD40L-mediated signaling inhibited PTX-mediated Th1 cell expansion and IFN- γ -dependent antibody production in the BALB/c background, and Th2 cytokine production on the C57BL/6 background. Overall, CD40L-mediated costimulation seemed not as critical for PTX-mediated adjuvant effects. Collectively, the data suggested that enhanced costimulatory signaling due to PTX-mediated activation of APCs was critical for the cytokine differentiation and clonal expansion of antigen-specific T cells. Furthermore, the data suggest an additive effect of cytokines for PTX-mediated adjuvant effects.

Activation and clonal expansion of T cells requires the encounter of specific antigen on MHC molecules presented by professional APCs. However, this signal, historically referred to as signal 1 [15], is not sufficient to induce T cell proliferation. Additional signals provided by several families of costimulatory molecules and cytokines are required to induce bona fide T cell activation, proliferation/expansion, and cytokine differentiation. Cytokine differentiation of Th1 and Th2 cells is driven by mutually exclusive conditions: differentiation towards Th1 cells is driven by IL-12 and IFN- γ and it is inhibited in the presence of Th2 type cytokines, such as IL-4. In contrast, IL-4 and IL-5 cooperate in the differentiation of T cells towards the Th2 pathway [16]. *In vivo* T cell activation and cytokine differentiation is promoted by adjuvants, such as CFA, which activate APCs and provide an antigen depot.

Activation of APCs by adjuvants is frequently mediated via the release of molecules that signal through TOLL-like receptors (TLRs), such as the stimulation of TLR4 by LPS. TLR4 signaling results in the release of IL-12 by APCs, which in turn favors Th1 cell cytokine differentiation. The signals mediating APC-induced Th2 differentiation are less well understood. However, IL-4 and IL-6, as well as antigen dose, are thought to play a role [17,18]. Also, a pathway involving the Notch family of ligands, which seems to be cytokine independent, has been described. Delta induces Th1 differentiation, while Jagged induces the alternate Th2 pathway [19].

PTX has well established adjuvant properties [7,8]. However, PTX differs from most classical adjuvants by its ability to concomitantly induce Th1 and Th2 cytokine responses [8]. The mechanism underlying the adjuvant activity of PTX, and specifically its propensity to enhance both Th1 and Th2 immune responses, has remained unresolved. Substantial evidence suggests that PTX exerts its adjuvant activity via the activation of APCs [12,20–22]. There is some

conflicting data on whether PTX acts by engagement of receptors on APCs via its PTX-B subunit [12], or whether PTX effects are mediated via the internalization of the G-protein active PTX-A subunit [10]. Recent data by Kerfoot et al. showed that the TLR4 also plays a role in the signaling of PTX [11,23]. TLR4 signaling ultimately results in translocation of NF- κ B, which induces transcription of a variety of genes, including genes for proinflammatory cytokines and P-selectin [24,25]. These findings can account for the effects of PTX on both Th1 and Th2 cells, as well as for its effect on the blood brain barrier described in EAE [26]. We have previously shown that the PTX preparations used in our studies contain only very low amounts of LPS that are not sufficient to mediate the observed adjuvant effects [12]. Hence, it is not likely that LPS, which is a potent TLR4 signaling molecule [11,23], played a role in our studies. PTX-activated APCs upregulate MHC II, and show enhanced production of cytokines such as IL-6 and IL-12 in vitro. Recently, Amend et al. showed that PTX upregulates the expression of CD80, CD86 and B7-DC on dendritic cells in vivo as well [27].

However, until now it has remained unresolved whether PT-mediated cytokine production or costimulation is more critical for T cell cytokine differentiation and expansion.

Our results address this issue by showing that blockade of CD28–CD80/86 costimulation essentially prevented PTX-mediated Th1 and Th2 expansion. CD80/86 blockade was most critical, and inhibition of both of these molecules together basically abolished antigen-specific Th1 and Th2 responses. Inhibition of either the CD80 or CD86 pathway alone was only partially effective in inhibiting the adjuvant function of PTX. Inhibition of costimulation by CD40L was substantially less critical in these studies and more variable and mouse strain dependent (C57BL/6 versus BALB/c mice).

A caveat to the interpretation of our results is that costimulatory blockade of CD80/86 might abrogate T cell activation in vivo per se, irrespective of PTX-mediated adjuvant effects. Indeed, it is well established that T cell activation is critically dependent on signals mediated via CD80/86 molecules. However, our data show that PTX-mediated cytokine production cannot overcome the lack of signaling via costimulatory molecules, thus establishing the critical link between PTX-mediated adjuvant effects and costimulatory molecules.

The cytokines tested in our studies were not obligatory for the adjuvant effects of PTX, but may have had some additive effects. Furthermore, cytokines could have redundant effects in PTX-mediated Th1/Th2 differentiation. This could potentially be addressed in future studies in mice deficient for several cytokines simultaneously. Also, it would be interesting to investigate the role of IL-17 and IL-23 in IL17- or p19-knockout mice.

Collectively, the data suggest that cytokines are redundant for the adjuvant activity of PTX. In contrast, signaling via costimulatory molecules was obligatory for PTX effects on T cell differentiation and expansion. Furthermore, the data suggest that PTX simultaneously affects multiple costimulatory pathways.

PTX could achieve this effect in several ways. First, PTX could modulate the function of extracellular receptors or intracellular adaptor molecules that regulate the activation state of APCs. For example, PTX binds to and modulates the function of TLR4 [11,23], which leads to upregulation of CD80 and CD86 and APC activation. As such, the NF- κ B pathway, which is situated downstream of TLRs, has multiple cell activating effects [25]. C3H-HeJ-mice, which carry a mutation in the toll-like receptor 4 gene, *Tlr4^{Lps-d}*, may be useful to address PTX effects on the TLR4 pathway in our model. Alternatively, a model involving the Notch family with the subsequent activation of T cells has to be considered [19].

Clearly, PTX-B mediated uptake of PTX-A into the cell also could result in APC activation, for example via inhibition of G-proteins. PTX-induced changes in intracellular calcium levels

may trigger the activation of kinases, such as the ras/JNK pathway, which could then lead to the activation of cfos and cjun and promote expression of costimulatory molecules and cytokine production [28,29].

However, PTX also could specifically modulate particular costimulatory pathways. As such, PTX could modulate elements upstream of costimulatory pathways, or it could affect molecules that are shared between different pathways. Alternatively, but not mutually exclusive, PTX could affect molecules unique to particular costimulatory pathways. At this point, it is speculative whether this is indeed the case. However, the reward to this answer may be novel insights into the modulation of cellular signaling pathways by microbial products. Importantly, it may eventually become feasible to selectively target the signals that trigger Th1 or Th2 differentiation with immunologic adjuvants.

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Abbreviations used in this paper

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

References

1. Mosmann TR, Cherwinski H, Bond MW, et al. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136(7):2348–57. [PubMed: 2419430]
2. Hsieh CS, Heimberger AB, Gold JS, et al. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc Natl Acad Sci U S A* 1992;89(13):6065–9. [PubMed: 1385868]
3. Macatonia SE, Hosken NA, Litton M, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 1995;154(10):5071–9. [PubMed: 7730613]
4. Yip HC, Karulin AY, Tary-Lehmann M, et al. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J Immunol* 1999;162(7):3942–9. [PubMed: 10201913]
5. Chu RS, Targoni OS, Krieg AM, et al. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 1997;186(10):1623–31. [PubMed: 9362523]
6. Munoz E, Zubiaga AM, Merrow M, et al. Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. *J Exp Med* 1990;172(1):95–103. [PubMed: 2162906]
7. Stewart SJ, Prpic V, Johns JA, et al. Bacterial toxins affect early events of T lymphocyte activation. *J Clin Invest* 1989;83(1):234–42. [PubMed: 2536043]
8. Hofstetter HH, Shive CL, Forsthuber TG. Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. *J Immunol* 2002;169(1):117–25. [PubMed: 12077236]
9. Linthicum DS, Munoz JJ, Blaskett A. Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. *Cell Immunol* 1982;73(2):299–310. [PubMed: 6891621]
10. Su SB, Silver PB, Zhang M, et al. Pertussis toxin inhibits induction of tissue-specific autoimmune disease by disrupting G protein-coupled signals. *J Immunol* 2001;167(1):250–6. [PubMed: 11418656]
11. Kerfoot SM, Long EM, Hickey MJ, et al. TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. *J Immunol* 2004;173(11):7070–7. [PubMed: 15557205]
12. Shive CL, Hofstetter H, Arredondo L, et al. The enhanced antigen-specific production of cytokines induced by pertussis toxin is due to clonal expansion of T cells and not to altered effector functions of long-term memory cells. *Eur J Immunol* 2000;30(8):2422–31. [PubMed: 10940934]
13. Ryan M, McCarthy L, Rappuoli R, et al. Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7-1, B7-2 and CD28. *Int Immunol* 1998;10(5):651–62. [PubMed: 9645613]
14. Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 1996;271(5256):1728–30. [PubMed: 8596934]
15. Lafferty KJ, Gill RG. The maintenance of self-tolerance. *Immunol Cell Biol* 1993;71(Pt 3):209–14. [PubMed: 8349303]
16. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996;17(3):138–46. [PubMed: 8820272]
17. Herrick CA, MacLeod H, Glusac E, et al. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J Clin Invest* 2000;105(6):765–75. [PubMed: 10727445]
18. Constant S, Pfeiffer C, Woodard A, et al. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med* 1995;182(5):1591–6. [PubMed: 7595230]

19. Amsen D, Blander JM, Lee GR, et al. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 2004;117(4):515–26. [PubMed: 15137944]
20. Olson RR, De Magistris MT, Di Tommaso A, et al. Mutations in the third, but not the first or second, hypervariable regions of DR(beta 1*0101) eliminate DR1-restricted recognition of a pertussis toxin peptide. *J Immunol* 1992;148(9):2703–8. [PubMed: 1573265]
21. Hofstetter HH, Sewell DL, Liu F, et al. Autoreactive T cells promote post-traumatic healing in the central nervous system. *J Neuroimmunol* 2003;134(1–2):25–34. [PubMed: 12507769]
22. Waldner H, Collins M, Kuchroo VK. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. *J Clin Invest* 2004;113(7):990–7. [PubMed: 15057305]
23. Racke MK, Hu W, Lovett-Racke AE. PTX cruiser: driving autoimmunity via TLR4. *Trends Immunol* 2005;26(6):289–91. [PubMed: 15922942]
24. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. *Science* 2003;300(5625):1524–5. [PubMed: 12791976]
25. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell* 2002;109(Suppl):S81–96. [PubMed: 11983155]
26. Brabb T, Goldrath AW, von Dassow P, et al. Triggers of autoimmune disease in a murine TCR-transgenic model for multiple sclerosis. *J Immunol* 1997;159(1):497–507. [PubMed: 9200491]
27. Amend B, Doster H, Lange C, et al. Induction of autoimmunity by expansion of autoreactive CD4 +CD62Llow cells in vivo. *J Immunol* 2006;177(7):4384–90. [PubMed: 16982873]
28. Deng T, Karin M. c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. *Nature* 1994;371(6493):171–5. [PubMed: 8072547]
29. Derijard B, Hibi M, Wu IH, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994;76(6):1025–37. [PubMed: 8137421]

Fig 1.

PTX-mediated enhancement of antigen-specific Th1 and Th2 cell responses is not dependent on specific cytokines. Female C57BL/6J wild type mice ($n=9$) or cytokine knockout mice ($n=4-7$ per group) for IL-4, IL-5, IL-6, or IL-12 on the C57BL/6 background were immunized with $100\ \mu\text{g}$ OVA:IFA subcutaneously, and $200\ \text{ng}$ PTX was injected at 0 and 24 hours. Three WT mice were immunized as above and injected with anti-IL-2 mAb ($0.5\ \text{mg}/\text{animal}$ i.p.). Shown is the antigen-induced production of IFN- γ (a), IL-2 (b), IL-4 (c), and IL-5 (d) by cytokine ELISPOT assay three weeks after the immunization with OVA:IFA and PTX. Similar results were obtained in two independent experiments. A star indicates statistical significance of results compared to controls ($p \geq 0.05$). A double star indicates p-value of less than 0.001. Results not marked with a star were not statistically significant ($p > 0.05$).

Fig 2.

PTX-mediated clonal expansion of Th1 and Th2 cells *in vivo* is costimulation dependent. Groups of female C57BL/6 mice were immunized with 100 μ g OVA:IFA s.c. (n=3 mice per group). 200 ng PTX was injected twice at 0 and 24 hours. One group was immunized only and served as a control. The other groups were treated with 0.5 mg per animal intraperitoneally of anti-CD80 mAb, anti-CD86 mAb, anti-CD40L mAb, or anti-CD80 and anti-CD86 mAb combined. The antibodies were injected three times, one week apart. Cytokine ELISPOT assay was performed three weeks after the immunization. Shown is the production of IFN- γ (a), IL-2 (b), and IL-5 (c) in the different treatment groups. The results are representative of three independent experiments. A star indicates statistical significance of results compared to controls ($p \geq 0.05$). A double star indicates p-value of less than 0.001. Results not marked with a star were not statistically significant ($p > 0.05$).

Fig 3.

Costimulation-requirement for PTX-driven T cell expansion is independent of particular antigen/mouse strain combinations. Groups of female BALB/c mice (n=3 mice per group) were immunized with 100 μ g HEL: Alum. 200 ng PTX was injected at 0 and 24 hours. One group was not treated further (control). The other groups were injected weekly (3 injections total) with 0.5 mg per mouse of anti-CD80 mAb, anti-CD86 mAb, anti-CD40L mAb, or anti-CD80 and anti-CD86 mAb combined. Cytokine ELISPOT assay was performed three weeks after the immunization. Shown is the production of IFN γ (a), IL-2 (b), and IL-5 (c) in the five different groups. The experiment was performed three times with the same results. A star indicates statistical significance of results compared to controls ($p \geq 0.05$). A double star indicates p-value of less than 0.001. Results not marked with a star were not statistically significant ($p > 0.05$).

Fig 4.

Costimulatory blockade decreases PTX-mediated CD4⁺ T cell expansion and cannot be reversed by naïve APCs in vitro. Female BALB/c mice were immunized with 100 μ g HEL:Alum, and 200 ng PTX at 0 and 24 hours. Three groups of mice (n=5) were injected with PBS (control), or with 0.5 mg per mouse of anti-CD40L mAb, or anti-CD80 and anti-CD86 mAb combined, three times, one week apart. Cytokine ELISPOT assay was performed three weeks after the immunization on purified CD4⁺ T cells as outlined in Methods. Irradiated APCs from naïve BALB/c mice (not treated with anti-CD40L or anti-CD80/86 antibodies) were used for the recall assay. Shown is the production of IFN- γ (a), IL-2 (b), and IL-5 (c) in the different groups. Similar results were obtained in two independent experiments. A double star indicates statistical significance of results compared to control ($p < 0.001$). Results not marked with a star were not statistically significant ($p > 0.05$).

Fig 5.

Costimulatory blockade prevents Th1/Th2 cytokine-mediated antibody class switching. Female BALB/c mice were immunized with 100 µg HEL in Alum, and 200 ng PTX was injected at 0 and 24 hours. Groups of mice (n=4) were injected i.p. with 0.5 mg per mouse of anti-CD40L mAb, anti-CD80 and anti-CD86 mAb combined, or left untreated. The antibodies were injected three times, one week apart. After three weeks serum was obtained for the detection of HEL-specific antibodies. Shown is the mean of 4 mice per group, tested in triplicates. Titration started at a serum dilution of 1:1500 and progressed in steps of 1:3. Error bars would have fallen within the size of the symbols. The results are representative of three experiments performed.