Published in final edited form as:

Cell Immunol. 2011 ; 267(1): 50–55. doi:10.1016/j.cellimm.2010.11.003.

Induction of polyclonal CD8+ T cell activation and effector function by Pertussis toxin

Cathi Murphey, Steve Chang, Xue Zhang, Bernard Arulanandam, and Thomas G. Forsthuber¹

Dept. Biology, University of Texas at San Antonio, TX 78249

Abstract

Pertussis toxin (PTX) has pronounced adjuvant activity and strongly enhances innate and adaptive immune responses, including increased antibody production and Th1/Th2 cytokine production. Adjuvant effects of PTX on Th1 and Th2 cells are primarily mediated via CD80/86 costimulation via enhanced expression of these molecules by APCs. However, it has remained unresolved whether PTX modulates the expression of costimulatory and inhibitory molecules on CD4+ and CD8+ T cells. To address this question, we determined the expression kinetics of CD28, CTLA-4, and CD40L on spleen CD4+ and CD8+ T cells after incubation with PTX. The results show that PTX upregulated the expression of CD28 by CD8+ T cells, but not by CD4+ T cells. In contrast, the expression of CTLA-4 and CD40L was not substantially altered on CD4+ or CD8+ T cells. CD28 upregulation by CD8+ T cells was paralleled by upregulation of CD69 and the induction of IFN- γ , Granzyme B (GrB), and IL-17. CD8+ T cell activation and cytokine production could be substantially blocked with anti-CD80 and CD86 antibodies, consistent with CD28 mediated signaling. Treatment of highly purified CD8+ T cells with PTX resulted in upregulation of CD28 and CD69, and production of IFN- γ . Incubation with CD28 mAb further enhanced this effect, suggesting that PTX has direct effects on CD8+ T cells which are enhanced by CD80/86-mediated costimulation provided by APCs.

Keywords

Pertussis toxin; T cell expansion; cytokines; costimulatory molecules

Introduction

Pertussis toxin (PTX) is an A–B type exotoxin secreted by *Bordetella pertussis*, the causative agent of whooping cough [1;2]. PTX has a wide range of biological effects including induction of leukocytosis, lymphocyte mitogenesis, enhancement of immunoglobulin IgG and IgE synthesis and strong adjuvant effects on antigen-specific T cell immunity promoting both Th1- and Th2-type inflammatory responses [3–8]. Of note, PTX is routinely used in experimental autoimmune disease models in animals, such as experimental autoimmune encephalomyelitis (EAE) in rodents, to promote the disease

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¹Correspondence: Thomas G. Forsthuber, Dept. Biology, University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249 United States, Tel: 210-458-5760, Fax: 210-4585499, thomas.forsthuber@utsa.edu.

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[9;10]. In EAE, the disease promoting effect of PTX was initially believed to be due to an increased permeability of the blood-brain-barrier for inflammatory lymphocytes [11;12]. Recent evidence suggests that in addition, PTX may promote autoimmune pathology by furthering the expansion and effector functions of pathogenic autoimmune T cells [13;14]. Exactly how PTX functions as an immune adjuvant remains unclear. Several studies showed that it activates APCs and upregulates CD80, CD86, and MHC class II molecules, suggesting that its effects on T cells are mediated indirectly [14]. Along these lines, Wakatsuki and colleagues provided evidence that PTX bound to APCs was critical for its T cell activating properties, and Denking and colleagues showed that PTX-induced clonal expansion and cytokine differentiation of Th1 and Th2 cells was primarily dependent on costimulation via CD80/86 molecules [15;16]. However, some studies have suggested a direct effect of PTX on T cells [17;18], and it is conceivable that modulation of CD28 and/or CTLA-4 expression on T cells could contribute to the PTX adjuvant activity.

Interaction of CD80 and CD86 on professional APCs with CD28 on T lymphocytes is critical for providing the “second signal” for activation of CD4+ T cells. The requirement of the CD80/CD86-CD28 costimulatory pathway for CD8+ T cells is more controversial. For example, the activation of naïve and memory CD8+ T cells is not impaired in the absence of CD28-mediated costimulation in some models, while the CD28 pathway is important for the formation of primary CD8+ T cell responses under certain conditions, such as in responses against DNA and peptides, for vaccination with dendritic cells (DC), and in the induction of antitumor CD8+ T cell immunity [19–21]. Furthermore, the CD80/CD86-CD28 pathway may be important for controlling the magnitude of memory CD8+ T cell responses against pathogens [22;23].

Signaling via CD28 molecules is negatively regulated by CTLA-4, which is upregulated upon T cell activation and subsequently outcompetes CD28 for binding to CD80/86 due to its higher binding affinity for these molecules [24;25]. Thus, it is conceivable that modulation of CD28 or CTLA-4 on T cells by PTX could be important for its adjuvant effects.

To begin to address this issue, we determined the expression of CD28, CTLA-4, and CD40L molecules on T cells upon stimulation with PTX. Unexpectedly, PTX strongly upregulated the expression of CD28 on CD8+ T cells, but not on CD4+ T cells. CD28 upregulation on CD8+ T cells was paralleled by upregulation of CD69 and induction of IFN- γ , Granzyme B (GrB) and IL-17. CD8+ T cell activation and cytokine production could be blocked with anti-CD80 and anti-CD86 antibodies, strongly implicating costimulation via CD80/86 molecules as the mode of PTX action.

Materials and Methods

Animals, antigens, and treatments

Female C57BL/6 mice (6 – 10 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the University of Texas at San Antonio (UTSA) under specific pathogen free conditions. All animal procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of UTSA. Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA) and used at the indicated concentrations. Blocking anti-CD80 (16-10A1) and anti-CD86 mAb (GL1) were gifts of Dr. Frederick Heinzel (Case Western Reserve University, Cleveland, OH). Anti-CD28 mAb was purchased from eBioscience (San Diego, CA). Anti-CD3 mAb was purified from hybridomas (2C11, ATCC). PMA and ionomycin was purchased from Sigma-Aldrich (St. Louis, MO). LPS (*E. coli* 0111:B4) was purchased from Sigma (St. Louis, MO).

Cell preparations from the spleen

Single cell suspensions from spleens were prepared as described previously [8]. The cells were counted and plated with antigen in DMEM complete medium (BioWhittaker, Walkersville, MD) (containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.2 mM L-glutamine) at 5×10^6 cells per well in 24 well plates, cultured, treated and tested as indicated in the text.

Cell separations

Following preparation of single cell suspensions from spleens, CD8⁺ T cells were purified with a FACSaria II cell sorter (BD Biosciences, San Jose, California). Flow cytometry analysis showed 99 – 99.5% enrichment for CD8⁺ cells.

Flow cytometry analysis

Single-cell suspensions were incubated at 1×10^6 cells per sample with 0.125 to 0.5 µg of anti-CD4, anti-CD8, anti-CD28, anti-CD40L, or anti-CTLA-4 mAbs, (eBioscience, San Diego, CA) for 30 min at 4°C in the dark. Cells were washed twice with PBS +2% FCS and fixed in IC Fixation Buffer (eBioscience). For intracellular cytokine staining by flow cytometry, the cells were restimulated with anti-CD3 mAb (10 µg/ml, plate immobilized) and anti-CD28 mAb (2 µg/ml, plate bound) for 5 h in the presence of Brefeldin A solution (3.0 µg/ml, eBioscience). Staining for cell-surface antigens was performed as described above. The cells were fixed with IC Fixation Buffer for 20 min, permeabilized with Permeabilization Buffer (eBioscience) and stained with 0.125 to 0.5 µg of anti-IFN-γ, anti-GrB, or anti-IL-17 mAbs for 30 min at RT in the dark. Samples were analyzed on a LSR II or FACSaria using BD FACS Diva software (BD Bioscience).

Statistical analysis

Statistical analysis was performed by two-tailed Student's t-test using SigmaStat software (Systat Software, San Jose, CA).

Results

Pertussis toxin upregulates CD28 expression on CD8⁺ T cells

To test the effect of PTX on the expression of costimulatory molecules on T cells, we cultured spleen cells from C57BL/6 mice with medium alone, or in the presence of plate bound anti-CD3 mAb, anti-CD3 mAb plus PTX, or PTX alone. The surface expression of CD28, CTLA-4, and CD40L molecules by CD4⁺ T cells was measured by flow cytometry as outlined in Materials and Methods.

As expected, incubation of spleen cells for 24 – 72 h with anti-CD3 mAb resulted in the upregulation of CD28 molecules by CD4⁺ T cells as compared with cells cultured in medium alone (Fig. 1A, top panels versus second row panels). Adding PTX to cultures with plate bound anti-CD3 antibody did not further enhance the expression of CD28 on CD4⁺ T cells (Fig. 1A, panels, third panels from top) and incubation with PTX alone showed only a modest increase in the expression of CD28 on CD4⁺ T cells after 72 h (Fig. 1A, bottom panels). However, we noted an approximately 10-fold increase in CD28 expression on a population of cells that was not CD4⁺ after 72 h incubation with PTX, as compared with the medium control (Fig. 1A, top versus bottom row; 1.2% versus 12.2%).

In contrast, the expression of CTLA-4 molecules under these experimental conditions was only modestly upregulated by anti-CD3 mAb on CD4⁺ T cells at the indicated time points, whereas CD40L expression was not affected (Fig. 1 B & C). Adding PTX to cultures with

plate bound anti-CD3 antibody did not substantially enhance the expression of either CTLA-4 or CD40L molecules on CD4⁺ T cells (Fig. 1 B & C, third panels from top). Furthermore, incubation of the cells with PTX alone had no effect on CTLA-4 or CD40L expression (Fig. 1 B & C, bottom panels). The expression of CTLA-4 and CD40L on cells not expressing CD4 was not affected by incubation with PTX alone (Fig. 1 B & C).

Overall, the results showed that PTX had relatively little effect on the expression of CD28, CTLA-4, and CD40L on CD4⁺ T cells. In contrast, PTX substantially enhanced CD28 expression by a population of cells that were not CD4⁺, and could conceivably consist of CD8⁺ T cells.

To further address this possibility, spleen cells were obtained from naïve C57BL/6 mice and incubated with medium alone, anti-CD3 mAb, PTX, or LPS for 72 h, and tested by flow cytometry for cell surface expression of CD28 on CD8⁺ T cells.

As shown in Fig. 2, PTX substantially enhanced the expression of CD28 on CD8⁺ T cells at 72 h, as compared with medium alone. Interestingly, at 72 h the effect of PTX on CD28 expression on CD8⁺ T cells was more pronounced than with anti-CD3 stimulation.

It has been suggested that PTX may act via TLR4 [26]. Therefore, we tested whether the effect of PTX on the upregulation of CD28 on CD8⁺ T cells was mimicked by LPS. However, incubation of spleen cells with LPS (10 – 100 ng/ml) did not enhance the expression of CD28 on CD8⁺ T cells (Fig. 2).

Taken together, the results show that PTX upregulated the expression of CD28 on CD8⁺ T cells, but had only a modest effect on CD28 expression on CD4⁺ T cells. In contrast, PTX did not substantially alter the expression of CTLA-4 or CD40L on either CD8⁺ or CD4⁺ T cells.

PTX induces cytokine production by CD8⁺ T cells

Next, we asked whether PTX-induced upregulation of CD28 on CD8⁺ T cells resulted in functional changes, such as cytokine production. To address this issue, spleen cells from C57BL/6 mice were incubated with medium alone, anti-CD3 mAb, or PTX and the production of IFN- γ , GrB, and IL-17 by CD8⁺ T cells was determined by intracellular cytokine staining by flow cytometry.

The results show that incubation with anti-CD3 mAb induced the production of IFN- γ , GrB, and IL-17 by CD8⁺ T cells, as compared with medium alone (Fig. 3A – C). Importantly, incubation of spleen cells with PTX induced substantial production of IFN- γ , GrB, and IL-17 by CD8⁺ T cells (Fig. 3A – C). PTX-induced production of IFN- γ by CD8⁺ T cells was most pronounced at 72 h, whereas anti-CD3 mAb-induced IFN- γ production peaked earlier (Fig. 3A). The kinetics of induction of GrB and IL-17 by PTX-incubated CD8⁺ T cells was comparable to that induced by stimulation with anti-CD3 mAbs.

Collectively, the results showed that induction of CD28 by PTX on CD8⁺ T cells was paralleled by production of IFN- γ , IL-17, and GrB.

PTX-induced CD8⁺ T cell activation is promoted via CD80/86 costimulation

The upregulation of CD28 on CD8⁺ T cells by PTX suggested that the CD80/86 pathway could play a role, similar to previous observations with CD4⁺ T cells [16].

To begin to address this possibility, C57BL/6 spleen cells were cultured with PTX in the presence or absence of blocking anti-CD80 mAbs, anti-CD86 mAbs, or both. Cells were

recovered after 72 h and the expression of CD28 molecules and cytokine production by CD8⁺ T cells was determined by flow cytometry.

The results show that blocking with anti-CD80 or anti-CD86 mAb decreased the PTX-induced expression of CD28 by CD8⁺ T cells, but the results did not achieve statistical significance (Fig. 4 A, $p > 0.05$). However, when anti-CD80 and anti-CD86 antibodies were combined, PTX-mediated upregulation of CD28 on CD8⁺ cells was significantly inhibited (Fig. 4A; $p < 0.05$). Similarly, neither anti-CD80 nor anti-CD86 mAb significantly decreased PTX-induced cytokine production (Fig. 4B). However, incubation of the cells with a cocktail of anti-CD80 and anti-CD86 mAb resulted in a $>35\%$ reduction of PTX-induced production of IFN- γ and GrB by CD8⁺ T cells (Fig. 4B). PTX-induced production of IL-17 was also reduced by anti-CD80/86 blockade, although not to a significant level (Fig. 4B).

Next, we asked whether the PTX-mediated increase in CD28 expression by CD8⁺ T cells was affected by soluble mediators, e.g. cytokines. However, incubation of naive spleen cells with supernatants from PTX-treated spleens, or with the cytokines IL-2, IL-7, or IL-15 did not enhance the expression of CD28 by CD8⁺ T cells (data not shown).

Taken together, the results showed that inhibition of CD80/86-mediated signals significantly attenuated the PTX-mediated increase in CD28 expression and production of cytokines by CD8⁺ T cells.

Direct effects of PTX on CD8⁺ T cells are enhanced by CD28-mediated costimulation

Our results suggested that PTX activated CD8⁺ T cells and induced cytokine production by a mechanism that involved CD80/86-mediated costimulation (Fig. 4). However, it has remained controversial whether PTX directly acts upon and activates CD4⁺ or CD8⁺ T cells.

Therefore, to address this question CD8⁺ T cells were isolated by FACS from the spleens of naïve C57BL/6 mice. The purified CD8⁺ T cells ($> 99\%$ purity) were incubated with anti-CD3 mAb, anti-CD3 plus anti CD28 mAb, PTX alone, or PTX plus anti-CD28 mAb for 24 – 72 h. The cell surface expression of the early activation marker CD69, CD28, and the production of IFN- γ were determined by flow cytometry and intracellular cytokine staining.

The results showed that incubation of purified CD8⁺ T cells with anti-CD3 mAb, or anti-CD3 mAb in combination with anti-CD28 mAb upregulated the expression of CD69 on CD8⁺ T cells and induced production of IFN- γ (Fig. 5A, B). Similarly, CD28 was upregulated (data not shown). Importantly, incubation of purified CD8⁺ T cells with PTX also enhanced the expression of CD69 and induced production of IFN- γ (Fig. 5A, B). Of note, incubation of CD8⁺ T cells with PTX in combination with anti-CD28 mAb substantially enhanced the expression of CD69 (Fig. 5A).

Together, the data show that PTX induced the upregulation of CD28, CD69 and production of IFN- γ by highly purified CD8⁺ T cells. The data support the notion that PTX has direct activating effects on CD8⁺ T cells. Furthermore, the data show that the effects of PTX on CD8⁺ T cells are amplified by costimulation mediated via CD28 molecules.

Discussion

In this study we show that PTX induced polyclonal activation and effector function of CD8⁺ T cells. Incubation with PTX upregulated the expression of CD28 and CD69 on CD8⁺ T cells and induced the production of IFN- γ , IL-17, and GrB. The PTX-mediated activation of CD8⁺ T cells was predominantly dependent on CD28 + CD80/86 costimulation and could

be substantially inhibited by anti-CD80/86 mAbs. The results also demonstrated that PTX had a direct effect on highly purified CD8+ T cells.

PTX is well known to exert *in vivo* adjuvant activity on CD4+ T cells, which is mediated via costimulatory molecules expressed by toxin-activated APCs [8;16]. However, relatively little is known about the regulation of costimulatory molecules expressed by CD4+ and CD8+ T cells by PTX.

Here, we show that PTX strongly upregulated CD28 on CD8+ T cells, but to a much lesser extent on CD4+ T cells. CD28 – CD80/86 signaling is well known to be critical for delivering “signal 2” for CD4+ T cell activation. In contrast, the requirement for CD28-mediated costimulation for CD8+ T cells is less well established. Thus, we asked whether the PTX-induced upregulation of CD28 on CD8+ T cells was paralleled by functional changes and our results showed that incubation with PTX induced strong production of IFN- γ , IL-17, and GrB by CD8+ T cells.

Interestingly, in our studies PTX was able to induce CD8+ T cell activation and effector function in the absence of TCR-mediated signaling, e.g. stimulation with anti-CD3 mAb.

These findings are consistent with observations by Wakatsuki and colleagues who reported CD4+ and CD8+ T cell proliferation and IFN- γ production after incubation with PTX [15]. Additionally, our results are in agreement with studies by Chen and colleagues showing PTX-induced cytokine production by CD4+ T cells [27]. The effects of PTX on cytokine production by CD4+ T cells reported by Chen and colleagues could be mimicked by TNF and IL-6, while the effects of PTX on CD8+ T cells in our studies required costimulation via CD28-CD80/86, as demonstrated by the fact that blocking anti-CD80/86 antibodies substantially inhibited PTX effects in contrast to incubation with PTX-conditioned medium or cytokines.

Previous studies on direct effects of PTX on CD4+ T cells showed conflicting results [15;18]. Wakatsuki and colleagues reported that PTX needs to be bound to APCs to activate T cells, whereas Schneider and colleagues reported that PTX induces direct signaling in Jurkat T cells; however, these cells are much less costimulation dependent [15;18]. Similarly, it has remained unresolved whether PTX has direct effects on CD8+ T cells.

Our data resolve these conflicting observations by showing that incubation of highly purified CD8+ T cells with PTX resulted in the upregulation of CD28 and CD69 and cytokine production. Importantly, PTX-mediated activation of purified CD8+ T cell activation was strongly enhanced when additional costimulatory signals were provided via stimulatory anti-CD28 antibodies. Furthermore, PTX-mediated activation of CD8+ T cells in spleen cell cultures could be substantially inhibited by blocking anti-CD80/CD86 mAbs, consistent with a previously reported role for these molecules in mediating PTX effects [8]. Thus, our data suggest a model where PTX signals directly to T cells. However, additional costimulatory CD28 - CD80/86 signals maximize the effects of the toxin, for example by PTX being directly bound to APCs as reported by Wakatsuki [15].

Along these lines, PTX has been reported to bind to and possibly signal through Mac-1, but a “PTX receptor” on T cells has not been identified [28;29]. Consistent with this model are the observations that the effects of PTX on CD8+ T cells are substantially inhibited by anti-CD80/CD86 antibodies, and that CD28-mediated signals enhance the PTX-mediated effects. It has been suggested that PTX activates APCs via signaling through TLR4 which could have accounted for its effects on CD8+ T cell activation [26;30;31]. Arguing against this view was our observation that incubation of spleen cells with LPS did not result in CD8+ T

cell activation. Thus, signaling through TLR4 alone did not seem to be sufficient to account for the effects of PTX on CD8+ T cells.

Work by several groups supports a role for PTX activating CD4+ T cells via the TCR or its downstream components including Lck, ZAP-70, and ERK pathway [17;18]. Conceivably, PTX could similarly activate CD8+ T cells using components of the TCR signaling pathway. However, this needs to be experimentally confirmed since signaling differences exist between CD4+ and CD8+ T cells, for example in CD45 isoforms associated with CD4 and CD8 molecules [32].

Our results raise the issue of the physiological role of polyclonal CD8+ T cell activation by PTX. It seems likely that this mechanism developed to provide a first line of defense to initiate the host immune response. However, it is unclear why PTX-induced T cell activation is costimulation dependent. Presumably, PTX provides an additional link between the innate and adaptive immune system [8;33].

Importantly, while polyclonal activation of CD8+ T cells could be beneficial for the immune defense against infectious diseases, it could be a concern with respect to autoimmune diseases. CD8+ T cells have been implicated in the pathogenesis of human autoimmune diseases [34;35] and have been shown to mediate pathology in animal models, including experimental autoimmune encephalomyelitis (EAE) and type I diabetes [36–38]. PTX-mediated polyclonal activation of CD8+ T cells could potentially result in the activation of pathogenic T cells and lead to autoimmune pathology. Alternatively, PTX could activate foreign-antigen specific CD8+ T cells to produce IL-17, which in turn could promote autoimmune disease in a bystander fashion. Along these lines, the well-known requirement for PTX in many animal models of autoimmune disease could be due to the induction of IL-17 by bystander CD8+ T cells. In addition, PTX could promote autoimmune pathology by inhibiting the function of regulatory T cells as suggested by reports that PTX inhibits CD4+ CD25+ T regulatory cells [39–41]. However, it has not been addressed whether the toxin affects CD8+ regulatory T cells. Overall, the induction of polyclonal CD8+ T cell activation by PTX and the release of proinflammatory cytokines may have important implications for the collaboration between the innate and adaptive immune systems. The effects of PTX should be considered in the development of vaccine adjuvants and in the pathogenesis of human autoimmune diseases.

Acknowledgments

We thank Cara Westbrook and Robert Day for excellent technical assistance and Drs. Neal Guentzel and Harvey Kaslow (University of Southern California) for helpful discussions.

This work was supported by grant NS-52177 from the National Institute of Health, and grants RG3499 and RG3701 from the National Multiple Sclerosis Society (T.G.F.).

Abbreviations

PTX	Pertussis toxin
GrB	Granzyme B
APC	antigen presenting cell

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Figure 1. PTX upregulates CD28, but not CTLA-4 or CD40L, on spleen cells

Pooled spleen cells from 3 – 5 C57BL/6 Wt mice were cultured for 24 – 72 h with medium, immobilized anti-CD3 mAb, immobilized anti-CD3 mAb plus PTX, or PTX alone.

Expression of CD28 (A), CTLA-4 (B), and CD40L (C) was measured by flow cytometry as outline in Material and Methods. Shown is the percentage of cells expressing the respective surface molecules in one representative experiment of three to five separate experiments with similar results.

Figure 2. PTX enhances the expression of CD28 on CD8+ T cells

Pooled spleen cells from 3 – 5 C57BL/6 Wt mice were cultured for 24 – 72 h with medium, immobilized anti-CD3 mAb, PTX, or LPS. Expression of CD28 on CD8+ T cells was analyzed by flow cytometry. Shown is the percentage of CD8+ T cells expressing the respective surface molecules. Shown are the means $\pm$ SD for three individual experiments.

Figure 3. PTX induces production of IFN-γ, GrB, and IL-17 by CD8+ T cells

Pooled spleen cells from 3 – 5 C57BL/6 mice were cultured for 24 – 72 h with medium, anti-CD3 mAb, or PTX alone. Expression of IFN-γ (A), GrB (B), or IL-17 (C) was determined by intracellular cytokine staining by flow cytometry as outline in Material and Methods. Data are shown as means ± SD of the percentage of gated CD8+ T cells expressing the indicated cytokines for three individual experiments.

Figure 4. PTX-mediated CD8+ T cell activation is dependent on CD80/86 costimulation
 Pooled spleen cells from 3 – 5 C57BL/6 mice were cultured for 24 – 72 h with anti-CD3 mAb or PTX in the presence of anti-CD80 mAb, anti-CD86 mAb, or both. (A) Shown is flow cytometry analysis of the expression of CD28 molecules on CD8+ T cells after the indicated treatments. * indicates statistical significance ($p < 0.05$). Results not marked were not statistically significant ($p > 0.05$). (B) Expression of cytokines by gated CD8+ T cells by intracellular cytokine staining by flow cytometry as outlined in Materials and Methods. Data represent the means $\pm$ SD of three independent experiments.

Figure 5. Activation of purified CD8+ T cells by PTX

CD8+ cells (>99% purity) were isolated from 3 – 5 C57BL/6 spleens by FACS as outline in Materials and Methods. Purified CD8+ T cells were cultured for 24 h to 72 h with medium, anti-CD3 mAb, or PTX, with or without plate-bound anti-CD28 mAb as indicated. Expression of CD69 and intracellular IFN- γ was determined by flow cytometry as outlined in Materials and Methods. (A) Expression of CD69 or (B) IFN- γ by purified CD8+ T cells after indicated treatments. Shown are means $\pm$ SD of one representative experiment of 4 independent experiments with similar results. A * indicates statistical significance by two-tailed t-test. (A) PTX versus Medium, $p=0.06$; PTX+anti-CD28 versus Medium, $p=0.04$; (B) PTX versus Medium, $p=0.0064$; PTX+anti-CD28 versus Medium, $p=0.023$. Results not marked with a star were not statistically significant ($p > 0.05$) or not determined.