Blockade of CTLA-4 (CD152) enhances the murine antibody response to pneumococcal capsular polysaccharides

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Abstract: The capsular polysaccharides (caps-PS) of *Streptococcus pneumoniae* are classified as thymus-independent antigens. Nevertheless, T lymphocytes can modulate the antibody response to caps-PS. In this study, we show that anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4) treatment, along with administration of caps-PS to BALB/c mice, resulted in a dose-dependent generation of a strong caps-PS-specific antibody response. Anti-CTLA-4 treatment had no effect on the immunoglobulin G (IgG) antibody production in athymic nu/nu mice. Anti-CTLA-4 treatment stimulated the IgG antibody production in severe combined immunodeficiency (*SCID*)/*SCID* mice reconstituted with CTLA-4$^{-/-}$ B lymphocytes and wild-type T lymphocytes. This excluded the possibility that anti-CTLA-4 enhanced antibody production by direct interaction with B lymphocytes. Anti-CTLA-4 treatment enhanced the antibody production in *SCID*/*SCID* mice reconstituted with B lymphocytes and CD4(+) and CD8(+) T lymphocytes but not in *SCID*/*SCID* mice reconstituted with B lymphocytes in the absence of CD4(+) and/or CD8(+) cells. Administration of anti-CTLA-4 in BALB/c mice but not in nu/nu mice resulted in a markedly increased production of interleukin (IL)-2, IL-4, and interferon-γ. Taken together, these data strongly suggest a role of T lymphocytes and CTLA-4 in the regulation of the antibody response to caps-PS. *J. Leukoc. Biol.* 78: 1060–1069; 2005.

Key Words: Streptococcus pneumoniae · *T lymphocytes*

INTRODUCTION

Streptococcus pneumoniae is a major cause of otitis media, pneumonia, and meningitis, especially in children, the elderly, and immunocompromised patients. The pneumococcal capsular polysaccharides (caps-PS) induce antibodies that provide clinical protection against invasive infections by these bacteria [1–3]. caps-PS are classified as thymus independent (TI) type 2 antigens for which T lymphocytes are not required for antibody production [4]. However, T lymphocytes regulate the magnitude of the antibody response to caps-PS. CD4(+) T lymphocytes have a positive effect, whereas CD8(+) T lymphocytes have a suppressive effect [5]. The molecular mechanisms of the T lymphocyte-mediated modulation of the anti-caps-PS immune response, however, are not understood.

Although it has been reported that blocking CD40 ligand (CD40L; CD154) did not influence the antibody response to caps-PS in mice immunized with isolated polysaccharides [6], we recently demonstrated that the antibody response to caps-PS was stimulated by CD4(+) T lymphocytes and inhibited by CD8(+) T lymphocytes and that these effects were mediated through the CD40-CD40L interaction [7, 8]. These findings, which point toward a physiologic role of the CD40-CD40L interaction in the antibody response to caps-PS, are in line with the demonstration that combined administration of an agonist anti-CD40 monoclonal antibody (mAb), which mimics CD40L activity with caps-PS, augments the anti-caps-PS response [9].

In addition to the CD40-CD40L interaction, the B7-CD28 interaction is a well-established costimulatory pathway in the immune response to protein antigens [10]. In antigen-specific T lymphocyte responses, activation signals are delivered through the T lymphocyte receptor and through binding of CD28 to B7-1 and/or B7-2 expressed on antigen-presenting cells (APC) [11]. The interaction between CD28 and B7-1 and/or B7-2 results in enhanced T lymphocyte proliferation and differentiation [12].

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152), a second receptor for the B7 molecules, fulfills a critical role in maintaining immunologic homeostasis [13]. CLTA-4 is a close relative of CD28 and binds B7-1 and B7-2 with an affinity ~20-fold higher than that of CD28 [14]. In contrast to CD28, which is constitutively expressed on T lym-

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phocytes, CTLA-4 is up-regulated rapidly after T lymphocyte activation and is a negative regulator of T cell activation [15]. Once CTLA-4 is up-regulated, the inhibitory B7-CTLA-4 interaction predominates, leading to the termination of T lymphocyte activation [13]. In addition to competing with CD28 for B7-1 and B7-2 binding, CTLA-4 might also inhibit the immune response by binding and dephosphorylating the phosphorylated T cell receptor (TCR)ζ, thereby antagonizing the TCR signal transduction [16], or by inhibiting downstream signaling of CD28 by suppression of nuclear factor-κB [17].

Only scarce information is available about the role of the CD28/CTLA-4-B7-1/B7-2 interaction in the antibody response to caps-PS. It has been reported that CTLA-4 immunoglobulin (Ig), a fusion protein that binds to the B7 molecules and prevents the interaction of these molecules with CD28 and CTLA-4, reduces the IgG response to phosphorylcholine, a TI type 2 antigen [18]. Similar reductions were noted in mice treated with anti-B7-2 mAb and in CD28-deficient mice [19]. In both studies, immunization was done using a nonencapsulated variant of type 2 *S. pneumoniae* (R36A). Whether and to what extent CTLA-4 fulfills a role in the regulation of the immune response to caps-PS are unknown.

The present study was undertaken to determine the role of CTLA-4 in the antibody response to caps-PS.

MATERIALS AND METHODS

Antigens and antibodies

Pneumovax®, a 23-valent pneumococcal vaccine containing 25 μg caps-PS (Danish nomenclature) types 1–5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F, was obtained from Aventis Pasteur MSD (Brussels, Belgium). C-polysaccharide and pneumococcal caps-PS types 3, 14, 19, and 22F were obtained from American Type Culture Collection (Manassas, VA). 4F10, a hamster anti-mouse mAb to CTLA-4, which blocks the B7-CTLA-4 interaction, was from Bioceros BV (Amsterdam, The Netherlands). Polyclonal hamster IgG and rat IgG2a antibodies were from Biotrend Chemikalien (Köln, Germany). The hamster IgG and rat IgG2a antibodies were dialyzed [buffer medium phosphate-buffered saline (PBS)] using a Spectra/Por Float-A-Lyzer (Spectrum Laboratories, Inc.) to remove the azides.

Mice

BALB/c mice were obtained from Centre d'élevage Janvier (Le Genest-St-Isle, France). CTLA-4$^{-/-}$/CD28$^{-/-}$ mice on BALB/c background were obtained from Roland J. Pieters (Utrecht, The Netherlands) and bred at the Catholic University Leuven (Belgium). Severe combined immunodeficiency (*SCID*/*SCID* mice were provided by Jan Mertens of the REGA Institute (Leuven, Belgium). Athymic *nu/nu* mice on BALB/c background were purchased from Harlan (Horts, The Netherlands). Male animals were used at the age of 6–8 weeks. BALB/c mice were kept under standard conditions with free access to pelleted food and water. *SCID/SCID*, CTLA-4$^{-/-}$/CD28$^{-/-}$, and *nu/nu* mice were kept in sterilized, plastic cages and were given sterilized, pelleted food and sterile tap water. The *SCID/SCID* mice were tested for leakiness by analyzing the level of mouse IgG antibodies according to a previously described enzyme-linked immunosorbent assay (ELISA) [20]. Only mice with IgG concentrations less than 2 μg/μl were used in the experiments. The local ethical committee of the Catholic University Leuven approved the study.

Immunization strategy

Mice were immunized with Pneumovax®. The vaccine was 1/25 diluted in NaCl 0.9%. This diluted vaccine (500 μl) was given intraperitoneally (i.p.). In experiments in which the effects of CTLA-4 were studied, 200 μg or 400 μg anti-CTLA-4 mAb 4F10 was injected i.p. 1 day before immunization with Pneumovax®. Hamster IgG control antibody (400 μg) was injected i.p. in the control group. After 3, 7, or 14 days, blood was drawn by intracardial puncture under ether anesthesia, and anti-caps-PS antibodies were detected by ELISA (see infra). In some experiments, mice were killed under ether anesthesia after 7 days, and spleens were harvested for cytokine measurements by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) or enzyme-linked immunospot (ELISPOT) assay (see infra).

ELISA for detection of polyclonal IgG antibodies

A MaxiSorp ELISA 96-well plate (Nunc, Nalge, Denmark) was coated overnight at 4°C with goat anti-mouse IgG (Sigma-Aldrich, Bornem, Belgium; final concentration 2 μg/ml in carbonate/bicarbonate buffer, pH 9.5). After coating, the plate was washed three times with 0.05% Tween 20 (Sigma-Aldrich) in PBS (Life Technologies, Paisley, UK). Serial dilutions of serum were made in PBS containing 2% goat serum (Life Technologies), which was added to the wells and incubated for 2 h at 37°C. After washing three times with 0.05% Tween 20 in PBS, peroxidase-conjugated goat anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) in a dilution of 1/5000 was added to the wells. The plate was incubated for 1.5 h at 37°C. Thereafter, 3,3′-5,5′-tetramethylbenzidine (Dako, Heverlee, Belgium) was added for color development. After 15 min, the reaction was stopped by an acid stock solution that contained 1 N HCl and 3 N H_2SO_4. Plates were read at 450 nm.

ELISA for detection of anti-cap-PS antibodies

Anti-caps-PS antibody levels were measured by ELISA as described previously [7]. Briefly, Covalink ELISA 96-well plates (Nunc) were coated overnight at 37°C with 3 μg/ml pneumococcal caps-PS of type 3, 14, or 19 in N-(3-dimethylaminopropyl)-N′-ethylcarbiimide hydrochloride (Merck, Darmstadt, Germany). After washing with 0.05% Tween 20 in PBS, the plates were blocked for 1 h at 37°C with 10% goat serum in PBS. Sera were treated with pneumococcal C-polysaccharide to remove anti-cell wall polysaccharide antibodies [21]. The plates were then incubated with serial dilutions of sera in duplicate for 2 h at 37°C. After washing, peroxidase-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, or IgG3 (Nordic Immunological Laboratories) in a dilution of 1/5000 was added to the wells. After incubation for 1.5 h at 37°C, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester was added for color development, and after 15 min, the reaction was stopped by an acid stock solution. Plates were read at 450 nm. Low absorbance values (<0.12) were found in mice before immunization.

It has been suggested that preabsorption of sera with caps-PS type 22F eliminates cross-reactive antibodies [22]. Therefore, we tested the effect of preabsorption with caps-PS 22F on the measurement of the antibodies to the serotypes used in this study (types 3, 14, and 19). Preabsorption with caps-PS type 22F (2 μg/ml) did not exert any effect on the anti-caps-PS (types 3, 14, and 19) antibody response in samples that had been preabsorbed with C-polysaccharide (n=4; data not shown).

Challenge of mice with *S. pneumoniae*

BALB/c mice were immunized with Pneumovax® and injected with 400 μg anti-CTLA-4 mAb 4F10 or polyclonal hamster IgG i.p. Three weeks later, the mice were challenged with 5 × 10^{E}5 colony-forming units (CFU) of encapsulated *S. pneumoniae* type 3, kindly provided by Jan Verhaegen, Department of Microbiology, University Hospital Leuven (Belgium). Numbers of surviving animals were ascertained 1 week after challenge.

Flow cytometry

Mice were killed under ether anesthesia, and the spleen was removed. Mouse mononuclear cells were isolated from the spleen using a cell strainer, 70-μm nylon (BD Labware Europe, Le Pont De Claix, France). Microscopic examination confirmed the mononuclear nature of the cell population. Erythrocytes were lysed in NH_4Cl^- buffer at 37°C for 15 min, and cells were washed with PBS. To 10^6 cells in 100 μL PBS, 15 μL fluorescein isothiocyanate (FITC)-labeled anti-mouse CD19 (1D3), FITC labeled anti-mouse CD4 (L3T4), or phycoerythrin-labeled anti-mouse CD8a (Ly-2) mAb (BD Biosciences, San Jose, CA) was added. The mixture was incubated for 10 min at room temperature. Cells were then washed twice with CellWASH (BD Biosciences) and fixed with CellFIX (BD Biosciences). Data on the percentage of B cells,

CD4(+) T cells, and CD8(+) T cells in the lymphogate were acquired with a FACSCalibur (BD Biosciences) by using the CellQuest program (BD Biosciences).

Transfer of spleen cells from BALB/c mice to *SCID/SCID* mice

Mouse mononuclear cells were isolated from the spleen, and the percentage of B lymphocytes, CD4(+) T lymphocytes, and CD8(+) T lymphocytes was determined by flow cytometry as described above. When indicated, CD4(+) T lymphocytes and/or CD8(+) T lymphocytes were removed by Dynabeads (Dynal Biotech, Hamburg, Germany). The cells were dissolved in 500 μL 0.9% NaCl and injected i.p. into *SCID/SCID* mice. On the same day, 400 μg anti-CTLA-4 mAb 4F10 or the isotype control hamster IgG was given i.p. The next day, Pneumovax® was administered. After 14 days, blood was drawn, and anti-caps-PS IgG was measured by ELISA.

Cytokine RT-PCR

RT-PCR for interleukin (IL)-2, interferon- γ (IFN-γ), IL-12, IL-4, IL-6, IL-10, transforming growth factor-β (TGF-β), and β-actin was performed as described previously [23]. Briefly, total RNA from splenocytes was extracted using the High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). A constant amount of 1 μg target RNA was reverse-transcribed using 100 U Superscript II RT (Life Technologies) at 42°C for 80 min in the presence of 5 μM oligo$(dT)_{16}$. PCR reactions were performed in the ABI Prism 7700 sequence detector (Perkin-Elmer/Applied Biosystems, Foster City, CA) using cytokine-specific primers and probes as described [24]. PCR amplifications were performed in triplicate. For each sample, normalization to β-actin was performed.

Cytokine ELISPOT assay

ELISPOT assays for the detection of IL-2-, IL-4-, and IFN-γ-secreting cells were performed using the ELISPOT SET from BD Biosciences, according to the manufacturer's instructions. Briefly, immunospot plates (Cellular Technology, Cleveland, OH) were coated overnight with 5 μg/ml anti-mouse IL-2, IL-4, or IFN-γ capture mAb in sterile PBS at 4°C. The plates were washed once with RPMI-1640 culture medium (Sigma-Aldrich). The plates were then blocked for 2 h with RPMI 1640 containing 10% fetal calf serum (FCS; Sigma-Aldrich) at room temperature. Freshly isolated splenocytes were suspended in complete RPMI 1640. Twofold dilutions of the cells were added to the wells in duplicate, starting with an amount of 10^6 cells per well. Cells were cultured for 48 h at 37°C in 5% CO_2. Medium containing no cells was used as negative control and medium containing 100 μg/ml phytohemagglutinin (Murex Diagnostics, Dartford, UK), as positive control. After washing two times with deionized water and three times with PBS containing 0.05% Tween-20 (PBST), 2 μg/ml biotinylated anti-mouse IL-2, IL-4, or IFN-γ mAb in PBS containing 10% FCS was added for 2 h at room temperature. The plates were then washed three times with PBST, followed by 2 h incubation with avidin-horseradish peroxidase in PBS containing 10% FCS at room temperature. After washing four times with PBST and twice with PBS, the plates were developed using the AEC substrate reagent set (BD Biosciences). The resulting spots, each representing a single cytokine-secreting cell, were counted under a dissection microscope and expressed as spots per 10^6 cells. The average number of spots/10^6 cells in the negative control ELISPOTs was 40 ± 10. The threshold for an ELISPOT response to be considered positive was 70/10^6.

Statistical analysis

Differences between the various conditions were calculated with the Wilcoxon-Mann-Whitney U-test (Analyze it for Microsoft Excel).

RESULTS

In vivo effect of anti-CTLA-4 treatment on the antibody response to pneumococcal caps-PS

To address the question of whether CTLA-4 fulfills a regulatory role in the generation of an antibody response to caps-PS of *S. pneumoniae* in vivo, the effect of anti-CTLA-4 treatment with the anti-CTLA-4 mAb 4F10 was studied. BALB/c mice immunized with Pneumovax® were treated with 4F10 or with a control hamster antibody. 4F10 administration was done 1 day before immunization with the vaccine. The immune response to serotypes 3, 14, and 19F was measured. Kinetic analysis revealed that the stimulation generated by anti-CTLA-4 administration was not yet obvious after 3 days, except for serotype 3, for which a small stimulation was observed. Seven days after anti-CTLA-4 treatment, a clear and significant stimulation was observed that was comparable with the stimulation found after 14 days (**Fig. 1**). The stimulation was more pronounced for serotype 3 than for serotypes 14 and 19F.

In the absence of 4F10, the vaccine induced an IgM and a weak IgG anti-caps-PS antibody response. Administration of 4F10 did not affect the IgM antibody response (data not shown) but markedly increased the IgG antibody response (caps-PS3 eightfold, caps-PS14 twofold, caps-PS19 twofold; Fig. 1). Injection of a control hamster antibody together with the vaccine did not affect the immune response to the vaccine (data not shown). The effect of anti-CTLA-4 treatment on the anti-caps-PS immune response was dose-dependent. A 2.3-fold increase of the specific anti-caps-PS immune response to serotype 3 was observed after administration of 200 μg 4F10, whereas an eightfold increase was observed after administration of 400 μg 4F10 (data not shown).

Administration of 4F10 not only induced high antibody titers, but it also resulted in isotype-switched responses. It significantly stimulated polysaccharide-specific IgG1, IgG2a, and IgG3 responses (**Fig. 2**). In the absence of 4F10, the vaccine mainly induced IgM and IgG3 anti-caps-PS antibodies.

Administration of 4F10 together with Pneumovax® also resulted in polyclonal activation of B cells with a resultant fourfold rise in total serum Ig levels (data not shown). This increase was significantly ($P<0.05$) less than the eightfold rise in polysaccharide-specific antibody production.

Neither mice treated with 4F10 nor mice treated with hamster IgG, without administration of Pneumovax®, mounted anti-caps-PS antibodies (data not shown).

Effect of anti-CTLA-4 treatment on survival after challenge with *S. pneumoniae*

To determine whether the advanced, isotype-switched, anti-caps-PS IgG antibody response induced by anti-CTLA-4 treatment had any functional significance, BALB/c mice, which had been immunized with Pneumovax® in the presence or absence of the anti-CTLA-4 mAb 4F10, were challenged i.p. with 5 × 10^E5 CFU *S. pneumoniae* type 3. Of the six mice to which 4F10 was administered together with the vaccine, five (83%) survived the *S. pneumoniae* challenge. By contrast, only one (17%) of six mice, which received the vaccine in the absence of 4F10, survived the *S. pneumoniae* challenge. Anti-CTLA-4 treatment in the absence of Pneumovax® did not protect the mice to a challenge with *S. pneumoniae*.

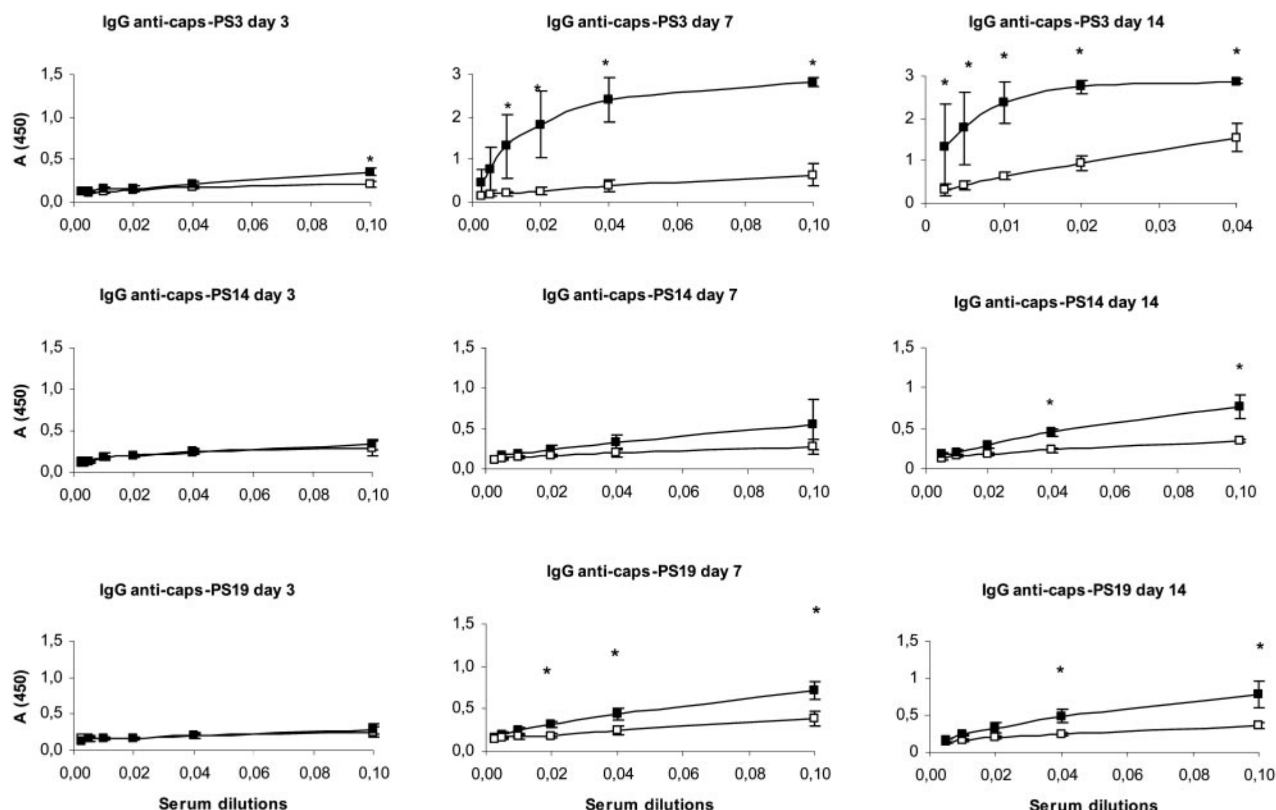

Fig. 1. Effect of anti-CTLA-4 treatment with 4F10 on the in vivo IgG immune response to caps-PS antigens. BALB/c mice were immunized with Pneumovax® on Day 0 and treated with 400 μg 4F10 (■) or hamster IgG (□) on Day –1. The IgG immune response to caps-PS types 3, 14, and 19 was measured 3, 7, and 14 days after immunization. The results show the absorbance values [A (450); mean±1 SD] for five or six serum dilutions. For serotype 3, each experimental group consisted of six animals. For serotypes 14 and 19F, each experimental group consisted of four animals. The data presented are from one representative of two independent experiments. *, Statistically, significantly different ($P<0.01$) by Wilcoxon-Mann-Whitney U-test.

The role of T lymphocytes in the CTLA-4-mediated regulation of the anti-caps-PS IgG response

The previous experiments suggested a key regulatory role for CTLA-4 in the antibody response to caps-PS. As CTLA-4 is expressed in T lymphocytes, a series of experiments was performed to test whether T lymphocytes, which have been shown to be involved in the regulation of the immune response to caps-PS [5], are involved in the CTLA-4-mediated modulation of the immune response to caps-PS.

In a first approach to study the role of T cells in the effects generated by the anti-CTLA-4 treatment on the anti-caps-PS immune response, the effect of the anti-CTLA-4 mAb 4F10 was studied in athymic *nu/nu* mice, which mounted a weak IgG anti-caps-PS antibody response not affected by CTLA-4 treatment (**Fig. 3**). These data suggested that T lymphocytes are necessary for CTLA-4-mediated regulation of the anti-caps-PS antibody response and excluded the possibility that anti-CTLA-4 mAb acted directly on B lymphocytes, natural killer cells, or monocytes.

In a second approach to exclude the possibility that anti-CTLA-4 treatment exerted its effect on the anti-caps-PS immune response by interacting directly with B lymphocytes rather than with T lymphocytes, we tested whether anti-CTLA-4 treatment augmented the anti-caps-PS immune response in *SCID/SCID* mice reconstituted with CTLA-4$^{-/-}$ and CD28$^{-/-}$ B lymphocytes and wild-type T lymphocytes. If the effect of anti-CTLA-4 treatment was a result of a direct effect on the B lymphocytes, then no stimulation would be seen under these conditions. The results are shown in **Figure 4**. Anti-CTLA-4 treatment augmented the anti-caps-PS immune response to several serotypes (3, 4, 14, and 19F) in *SCID/SCID* mice reconstituted with CTLA-4$^{-/-}$ and CD28$^{-/-}$ B lymphocytes and wild-type T lymphocytes. This excluded the possibility that anti-CTLA-4 mAb exerted its effect by acting directly on B lymphocytes.

Next, we investigated whether CD4(+) T lymphocytes or CD8(+) T lymphocytes were implicated in the CTLA-4-mediated effects on the anti-caps-PS immune response (**Fig. 5**). *SCID/SCID* mice were reconstituted with total spleen cells, splenocytes depleted of CD4(+) cells [leaving a B lymphocyte fraction and CD8(+) T lymphocytes], splenocytes depleted of CD8(+) cells [leaving a B lymphocyte fraction and CD4(+) T lymphocytes], or splenocytes depleted of CD4(+) and CD8(+) T lymphocytes (leaving a B lymphocyte fraction). Pneumovax® was given, and the effect of the anti-CTLA-4 treatment on the antibody formation to caps-PS was assessed. In the absence of anti-CTLA-4, no measurable anti-caps-PS antibodies were found, except for the condition, in which *SCID/SCID* mice were reconstituted with a B cell fraction, and CD4(+) T cells, in which a weak antibody response was observed. Anti-CTLA-4 treatment resulted in a clear rise of the anti-caps-PS IgG

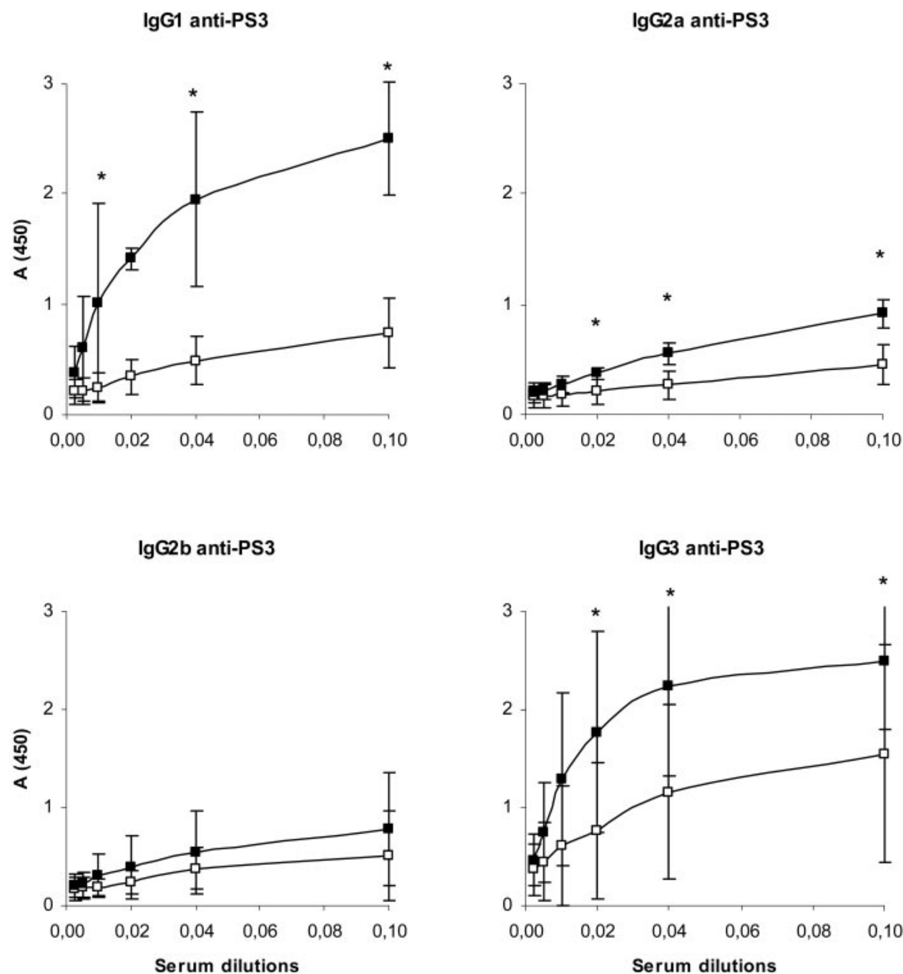

Fig. 2. Effect of anti-CTLA-4 treatment with 4F10 on the in vivo IgG subclass immune response to caps-PS antigens. BALB/c mice were immunized with Pneumovax® on Day 0 and treated with 400 μg 4F10 (■) or hamster IgG (□) on Day –1. The IgG subclass immune response to caps-PS type 3 was measured 14 days after immunization. The results show the absorbance values (mean±1 SD) at various serum dilutions. Each experimental group consisted of six animals. The data presented are from one representative of two independent experiments. *, Statistically, significantly different ($P<0.01$) by Wilcoxon-Mann-Whitney U-test.

antibody levels in *SCID/SCID* mice reconstituted with total spleen cells but not in *SCID/SCID* mice reconstituted with spleen cells depleted of CD4(+)and/or CD8(+) cells. These results indicated that the CTLA-4-mediated regulatory effects on the anti-caps-PS immune response were dependent on the presence of CD4(+) T lymphocytes as well as CD8(+) T lymphocytes. In the condition in which only B cells were transferred to *SCID/SCID* mice, no effect of anti-CTLA-4 was observed.

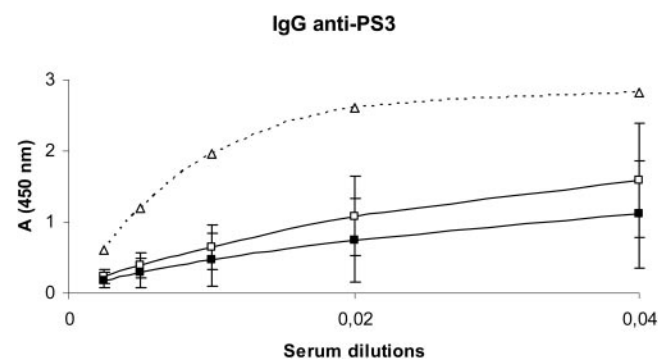

Fig. 3. Effect of anti-CTLA-4 treatment with 4F10 on the in vivo IgG immune response to caps-PS in athymic *nu/nu* mice, which were immunized with Pneumovax® on Day 0 after treatment with 400 μg 4F10 (■) or hamster IgG (□) on Day –1. The IgG immune response to caps-PS type 3 was measured 14 days after immunization. The results show the absorbance values (mean±1 SD) at various serum dilutions. Each experimental group consisted of six animals. A serum pool obtained from BALB/c mice (n=8) immunized with Pneumovax® (—△—) was analyzed as well. Differences between the 4F10- and hamster IgG-treated *nu/nu* mice were not statistically significant (Wilcoxon-Mann-Whitney U-test).

The effect of anti-CTLA-4 treatment and caps-PS immunization on cytokine mRNA expression and cytokine production

As it has been reported that signaling through the CTLA-4 receptor influences the pattern of cytokines produced by T cells [13], we studied changes in the production of cytokines as a result of anti-CTLA-4 treatment during the anti-caps-PS immune response. As immunohistochemical studies described peak numbers of cytokine-producing cells 5–7 days after immunization with a TI type 2 antigen (trinitrophenol-Ficoll), cytokines were measured 7 days after immunization [25]. BALB/c mice were immunized with Pneumovax®, and expression of murine IL-2, IFN-γ, IL-12, IL-4, IL-6, IL-10, and TGF-β genes was measured by real-time RT-PCR. In preliminary studies, we found that no measurable mRNA was detected, unless cells were restimulated in vitro with caps-PS. Under these condition, immunization of mice with Pneumovax® in the presence of the anti-CTLA-4 mAb 4F10 resulted in the up-regulation of IL-2, IL-4, and IFN-γ transcription (**Fig. 6**) but not of IL-12, IL-6, IL-10, and TGF-β transcription (data

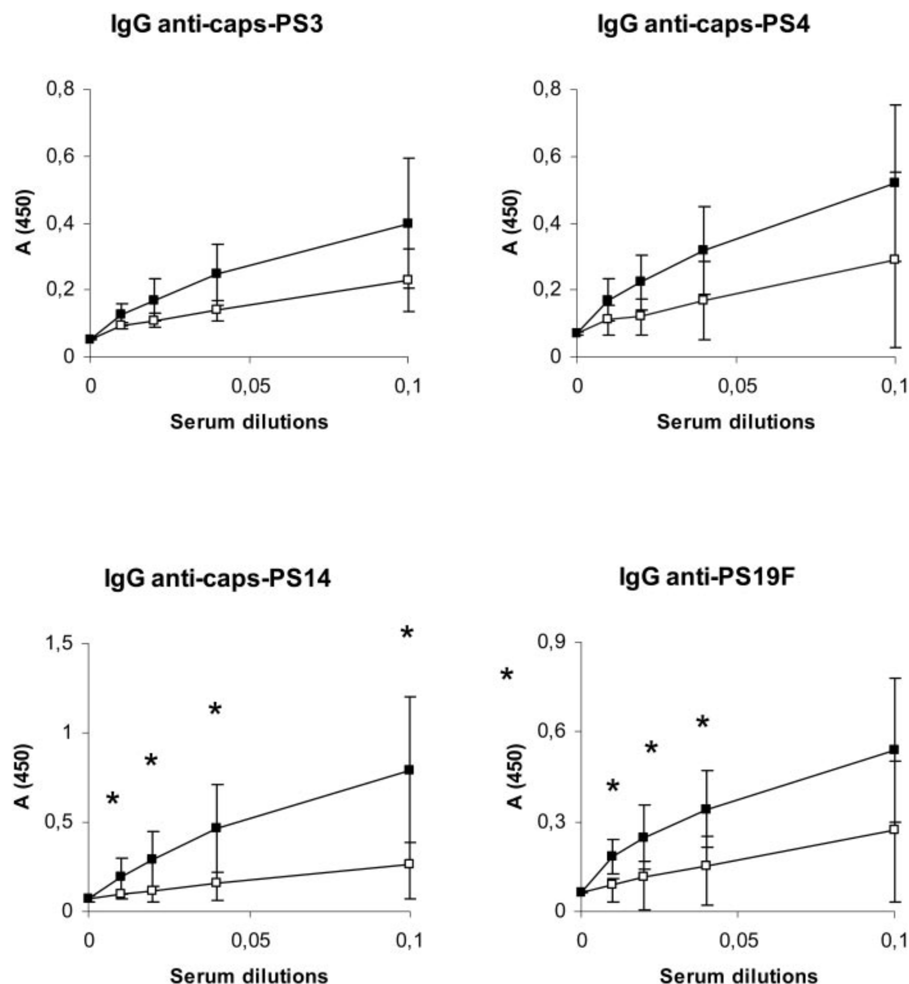

Fig. 4. Effect of anti-CTLA-4 treatment with 4F10 on the in vivo IgG immune response to caps-PS in *SCID/SCID* mice reconstituted with wild-type T lymphocytes and CTLA-$4^{-/-}$ B lymphocytes. *SCID/SCID* mice were reconstituted with B cells from CTLA-$4^{-/-}$/CD$28^{-/-}$ mice and with T cells from wild-type BALB/c mice. The B cells were obtained from spleen after depletion of CD4(+) and CD8(+) cells. The T cell fraction was obtained by depleting the B lymphocytes. The cells were suspended in 0.9% NaCl and injected i.p. into *SCID/SCID* mice. The total amount of B lymphocytes was the same for all mice and amounted to $\pm 10 \times 10^6$. Depletion of CD4(+) and/or CD8(+) cells resulted in cell fractions containing <1.5% CD4(+) and/or CD8(+) T lymphocytes. Depletion of B lymphocytes resulted in cell fractions containing <1.8% B lymphocytes ±52% CD4(+) and ±12% CD8(+) cells. The day after reconstitution, mice were immunized i.p. with Pneumovax® in the presence (■) or absence (□) of the anti-CTLA-4 mAb 4F10 (400 μg). The IgG immune response to pneumococcal caps-PS types 3, 4, 14, and 19F was measured 14 days after immunization. The results show the mean absorbance values at various serum dilutions. The data presented are mean and SD from three independent experiments. *, Statistically, significantly different ($P<0.05$) by Wilcoxon-Mann-Whitney U-test.

not shown). In a second approach, we quantified the number of IL-2-, IL-4-, and IFN-γ-secreting splenocytes by an in vitro ELISPOT assay. Mice were immunized with Pneumovax® in the presence or absence of 4F10, and IL-2-, IL-4-, and IFN-γ-secreting splenocytes were measured after 7 days. Anti-CTLA-4 treatment resulted in a marked increase of the number of IL-2-, IL-4-, and IFN-γ-secreting splenocytes (**Fig. 7**). No differences in the number of cytokine-secreting cells were found between mice immunized with Pneumovax® and control mice, which had not been immunized, and between mice immunized with Pneumovax® and mice immunized with Pneumovax® and a control hamster antibody (data not shown). Anti-CTLA-4 treatment in the absence of Pneumovax® also resulted in an increase of cytokine-producing cells (Fig. 7).

Administration of 4F10 to *nu/nu* mice immunized with Pneumovax® did not affect the number of IL-2-, IL-4-, and IFN-γ-producing splenocytes (data not shown). In *nu/nu* mice, almost no IL-2- and only small numbers of IL-4- and IFN-γ-secreting splenocytes were present, providing evidence that T lymphocytes were the major source of these cytokines in our experimental model.

DISCUSSION

The polysaccharide capsule is the principal determinant of the virulence of *S. pneumoniae*. Protection after immunization is dependent on production of circulating type-specific anti-caps-PS antibodies [1–3]. caps-PS are TI type 2 antigens and do not require the presence of mature T lymphocytes to elicit an antibody response. However, T lymphocytes can modulate the anti-caps-PS immune response. CD4(+) T lymphocytes augment the antibody response, whereas CD8(+) T lymphocytes suppress the antibody response [5]. Recently, we reported an essential role for CD40-CD40L interactions in T lymphocyte-mediated modulation of the immune response to pneumococcal caps-PS [7, 8].

In the present report, we describe that the in vivo antibody response to various caps-PS antigens could be modulated markedly by anti-CTLA-4 treatment. Administration of the anti-CTLA-4 mAb 4F10, which blocks the B7-CTLA-4 interaction, resulted in an enhanced IgG response to caps-PS. The antibodies that were generated were protective against lethal infection with *S. pneumoniae*. Although an increase in polyclonal IgG was observed in mice treated with 4F10, this increase was smaller than the increase of anti-caps-PS-specific antibodies. Furthermore, administration of 4F10 without caps-PS did not result in an increase in anti-caps-PS antibody production, confirming the fact that 4F10, when given in the presence of caps-PS, specifically stimulates the anti-caps-PS antibody response.

Kinetic analysis of the anti-caps-PS antibody response showed that the effect of anti-CTLA-4 treatment could already be seen 7 days after immunization. These findings are consis-

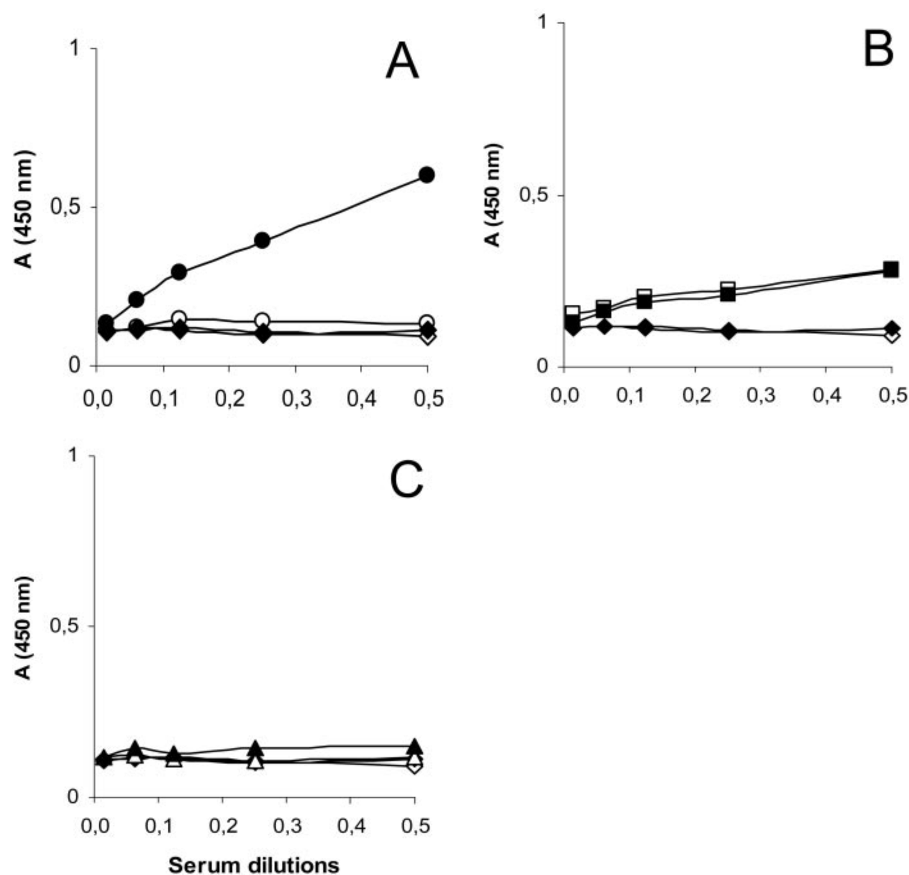

Fig. 5. Effect of anti-CTLA-4 treatment with 4F10 on the in vivo IgG immune response to caps-PS in reconstituted *SCID/SCID* mice, which were reconstituted with total spleen cells from BALB/c mice (A, circles) or with splenocytes depleted of CD4(+) T lymphocytes [leaving a B lymphocyte fraction and CD8(+) T lymphocytes; C, triangles]; CD8(+) T lymphocytes [leaving a B lymphocyte fraction and CD4(+) T lymphocytes; B, squares]; or CD4(+) and CD8(+) T lymphocytes [leaving a B lymphocyte fraction; A–C, diamonds] in the presence (filled symbols) or absence (open symbols) of the anti-CTLA-4 mAb 4F10 (400 μg). The cells were suspended in 0.9% NaCl and injected i.p. into *SCID/SCID* mice. The total amount of B lymphocytes was the same for all mice and amounted to $\pm 15 \times 10^6$. Total spleen cells consisted of a mean of 48% (range 44–50%) B lymphocytes, 33% (range 28–34%) CD4(+) T lymphocytes, and 12% (range 14–19%) CD8(+) T cells. Depletion of CD4(+) and/or CD8(+) T lymphocytes resulted in cell fractions containing <1.5% CD4(+) and/or CD8(+) T lymphocytes. The day after reconstitution, mice were immunized i.p. with Pneumovax®. The IgG immune response to pneumococcal caps-PS type 3 was measured 14 days after immunization. The results show the mean absorbance values at various serum dilutions. The data presented are from one representative out of four independent experiments.

tent with the data of Snapper and co-workers [18], who showed that after immunization of mice with a noncapsulated variant of *S. pneumoniae*, anti-C-polysaccharide antibodies reached maximum levels by Day 7, in contrast to the anti-PspA antibodies, which reached a maximum after 14 days.

CTLA-4 has been reported to be expressed not only on T lymphocytes but also on B lymphocytes [26] and monocytes [27]. Several experimental approaches were used to find out which cell types were critically involved in the CTLA-4-mediated regulation of the anti-caps-PS immune response. First, we showed that anti-CTLA-4 treatment exerted no effect on the anti-caps-PS immune response in *nu/nu* mice. This suggested that T lymphocytes played a crucial role in the CTLA-4-mediated effect on the anti-caps-PS immune response.

Second, we found that anti-CTLA-4 treatment stimulated the IgG antibody production in *SCID/SCID* mice reconstituted with CTLA-$4^{-/-}$ B lymphocytes and wild-type T lymphocytes. This excluded the possibility that CTLA-4, which has been reported to be expressed on B lymphocytes [26], acted via direct interaction with B cells.

Next, we found that anti-CTLA-4 treatment clearly augmented the IgG anti-caps-PS antibody levels in *SCID/SCID* mice reconstituted with total spleen cells but not in *SCID/SCID* mice reconstituted with spleen cells depleted of CD4(+) cells and/or CD8(+) cells. These results indicated that the CTLA-4-mediated regulatory effects on the anti-caps-PS-immune response were dependent on the presence of CD4(+) T lymphocytes and CD8(+) T lymphocytes. The observation that anti-CTLA-4 treatment did not affect the immune response in *SCID/SCID* mice reconstituted with B lymphocytes without T lymphocytes confirmed that the effect of blocking CTLA-4 was not through direct interaction with B lymphocytes or monocytes.

One proposed mechanism for regulation of T cell responses by CTLA-4 is through the function of $CD25^+CD4^+$ regulatory T cells. This idea is based largely on the finding that regulatory T cells express high amounts of CTLA-4 [28]. We tested whether administration of anti-CD25 mAb (PC61) abolished the effect of anti-CTLA-4 treatment. It is well known that anti-CD25 treatment results in a reduction of $CD4^+CD25^+$ cells in the peripheral tissues [29]. We found that after depletion of $CD25^+$ cells, anti-CTLA-4 treatment failed to augment the antibody response to caps-PS (manuscript in preparation). These findings indicate that $CD4^+CD25^+$ immunoregulatory cells were involved in the stimulatory effect of anti-CTLA-4 treatment.

Our results, based on a series of complementary experiments, illustrate that the anti-CTLA-4 mAb does not exert its effect by interacting directly with B lymphocytes or with monocytes. The effect is T cell-specific. However, we cannot exclude that monocytes are involved in the T cell-mediated effect of anti-CTLA-4 treatment via an indirect mechanism.

Our observation—that the stimulatory effect of anti-CTLA-4 mAb was observed in the condition in which B lymphocytes with CD4(+) and CD8(+) T lymphocytes were present but not in the condition in which only B lymphocytes and CD4(+) T

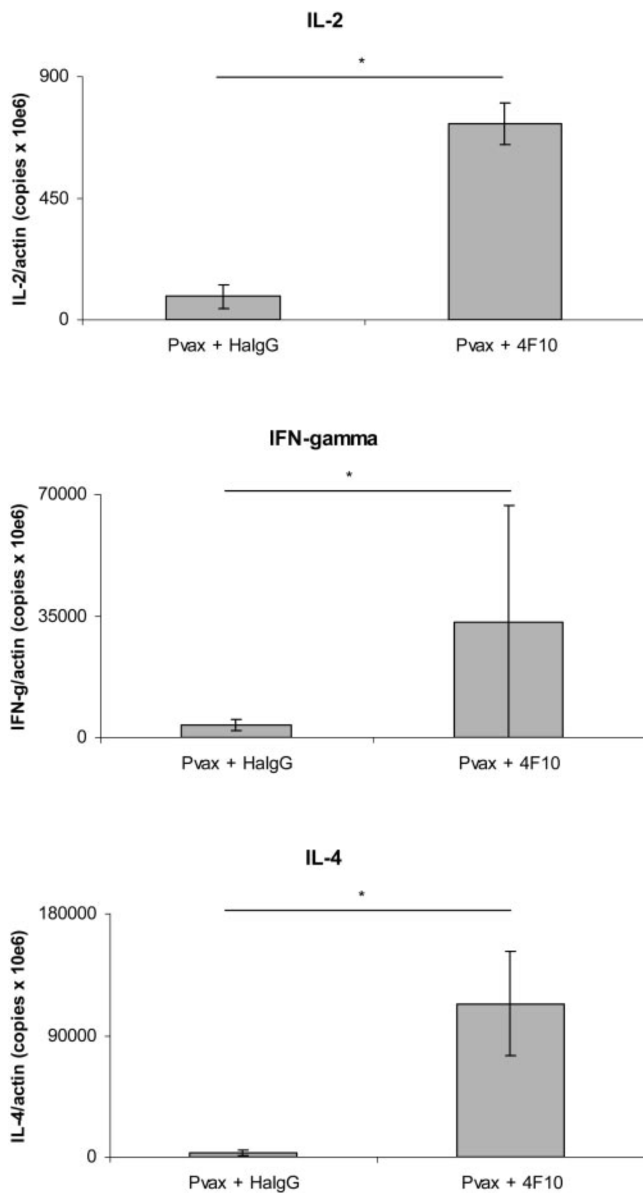

Fig. 6. Effect of anti-CTLA-4 treatment with 4F10 on the expression of various cytokine genes following immunization with caps-PS. BALB/c mice were immunized with Pneumovax® (Pvax) on Day 0 after treatment with 400 μg 4F10 or hamster IgG (HaIgG) on Day –1. On day 7, splenocytes were isolated and cultured for 4 h in the presence of caps-PS type 19. IL-2, IFN-γ, and IL-4 mRNA expression was determined by RT-PCR. Data are presented as the mean mRNA level per cytokine relative to the β-actin expression [(number of cytokine copies/number of β-actin copies)$\times 10^6$] for each sample. Each experimental group consisted of four animals, and each condition was done in triplicate. *, Statistically, significantly different ($P<0.05$) by Wilcoxon-Mann-Whitney U-test.

lymphocytes were present—suggested an important role not only for CD4(+) T lymphocytes but also for CD8(+) T lymphocytes in the CTLA-4-mediated modulation of the anti-caps-PS antibody response. Even if many studies about the function of CTLA-4 have focused on CD4(+) T lymphocytes, a role for CD8(+) T lymphocytes in the CTLA-4-mediated regulation of the immune function has been suggested. Although studies using CD8(+) T lymphocytes from CTLA-4-deficient mice gave contradictory results, studies using the anti-CTLA-4 mAb 4F10 supported such a view [30, 31]. In a model of murine chronic graft-versus-host disease, in which CD8(+) T lymphocytes are thought to play a regulatory role, treatment with anti-CTLA-4 mAb enhanced CD8(+) donor T lymphocyte expansion [32]. Also, primary T lymphocyte responses, induced by immunization with lymphocytic choriomeningitis virus antigens on dendritic cells in vivo, were enhanced by treatment with anti-CTLA-4 mAb [33]. Finally, in several tumor models, the administration of anti-CTLA-4 mAb was

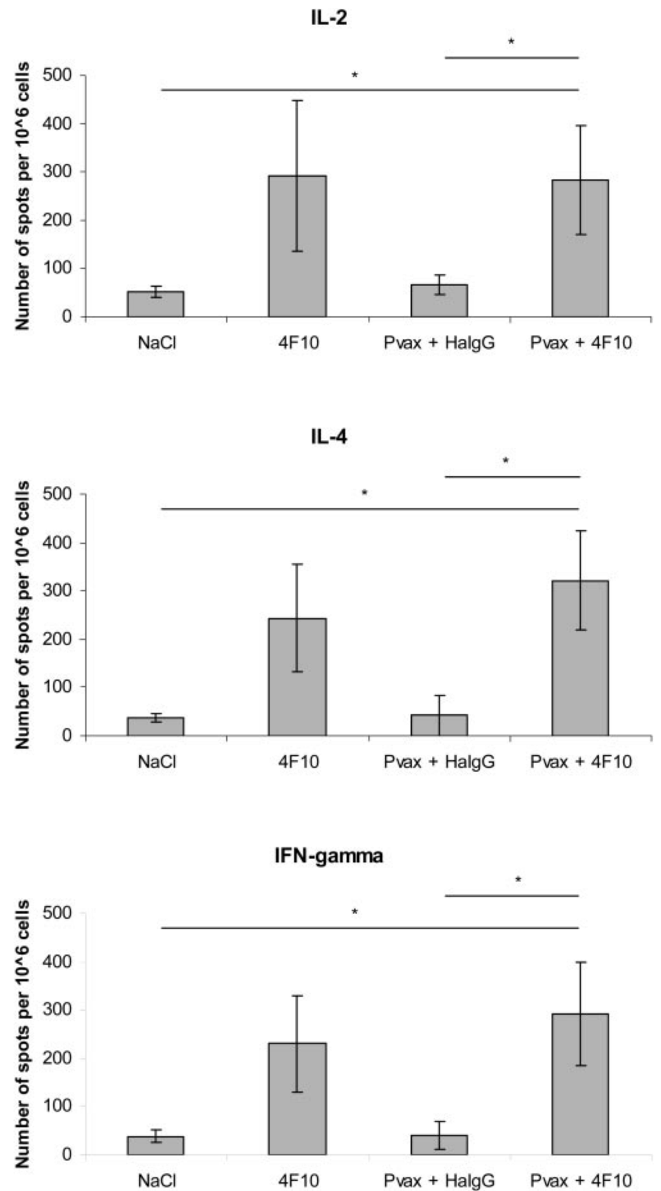

Fig. 7. Effect of anti-CTLA-4 treatment with 4F10 on the frequency of IL-2-, IL-4-, and IFN-γ-producing spleen cells following immunization with caps-PS. BALB/c mice were immunized with Pneumovax® on Day 0 after treatment with 400 μg 4F10 (Pvax + 4F10) or hamster IgG (Pvax + HaIgG) on Day –1. Two additional control groups were injected with 4F10 or NaCl 0.9% on Day –1. Mice were killed on Day 7, and freshly harvested splenocytes were analyzed for IL-2, IL-4, and IFN-γ production by ELISPOT assay. Each condition was done in triplicate. The results show the number of spots per 10^6 cells (mean±1 SD). Each experimental group consisted of four animals. *, Statistically, significantly different ($P<0.05$) versus NaCl, Pvax + HaIgG by Wilcoxon-Mann-Whitney U-test.

necessary to obtain tumor rejection by CD8(+) T lymphocytes after tumor cell vaccination [34–37]. In some experimental tumor models, CD4(+) T lymphocytes were required for the CD8(+) T lymphocyte expansion to occur [34, 35], whereas in other experimental tumor models, the enhanced effect of anti-CTLA-4 treatment was seen, irrespective of the presence or absence of CD4(+) T lymphocytes [36, 37].

In our experiments, anti-CTLA-4 mAb were used, and therefore, we cannot rule out the possibility that the effects observed arose from nonphysiological mechanisms rather than from blocking the physiological interaction between CTLA-4 on T cells and B7 on APC. For example, Walunas et al. [14] found that T cell proliferation was augmented to a lesser extent by CTLA-4 blocking Fab fragments compared with blocking mAb. These data suggested that T lymphocyte signaling was important in addition to the assumed mechanism of interrupting the physiological signals delivered through CTLA-4.

The enhanced antibody response to caps-PS observed after anti-CTLA-4 treatment was associated with an increased production of IL-2, IL-4, and IFN-γ by T lymphocytes. The major effect of 4F10 on cytokine production was polyclonal, as administration of 4F10 without Pneumovax® resulted in a significant increase of IL-2, IL-4, and IFN-γ production. The effect of 4F10 on the anti-caps-PS antibody response, conversely, was specific, as mice treated with 4F10 without Pneumovax® did not generate anti-caps-PS antibodies and had a high mortality after challenge with *S. pneumoniae*. This pattern of cytokine up-regulation, with increased levels of T helper cell type 1 (Th1) and Th2 cytokines, has also been described in other experimental models in which anti-CTLA-4 treatment was used. For example, it was demonstrated that anti-CTLA-4 treatment resulted in enhanced production of IL-2, IFN-γ, and IL-4 by superantigen-reactive T lymphocytes [38]. Besides, in an experimental model in which mice were infected with *Leishmania donovani*, a mixed Th1 and Th2 cytokine profile was enhanced by anti-CTLA-4 treatment [39]. It has been suggested that the effects of CTLA-4 on T lymphocyte cytokine production are dependent on the context under which T lymphocyte activation occurs and are not a result of an inherent capability of CTLA-4 [13].

As administration of anti-CTLA-4 mAb in the absence of Pneumovax® also resulted in increased cytokine production, one cannot rule out the possibility that T lymphocyte activation through auto- or alloantigens from the environment was responsible for the observed increase in cytokines. The increased cytokine production after administration of anti-CTLA-4 could contribute to the enhancement of the immune reaction to caps-PS.

The role of cytokines in the CTLA-4-mediated effect on the anti-caps-PS immune response is currently under investigation in our laboratory. Recently, we found that anti-CTLA-4 treatment failed to enhance antibody production in IFN-γ receptor knockout mice (manuscript in preparation), which indicated that IFN-γ was essential in the effects generated by anti-CTLA-4.

Our findings also point toward a substantial potential of anti-CTLA-4 treatment as an immunological adjuvant for polysaccharide antigens. Classically, polysaccharide vaccines only generate weak antibody responses. Therefore, recent vaccines and most vaccines under development for use against encapsulated bacteria are polysaccharide-protein conjugates. Epitopes on the protein part of the vaccine are recognized by T cells, which provide help for the anti-caps-PS response. We addressed the question of whether the antibody response to the conjugacted vaccine (Prevenar®) was equivalent to the antibody response to Pneumovax® administered together with 4F10. Prevenar® generated a greater antibody response to caps-PS types 19 and 14 than Pneumovax® administered together with 4F10 (data not shown). However, this comparative analysis could not be done for serotype 3 (to which the antibody response was most stimulated by anti-CTLA-4 treatment), as it is not contained in Prevenar®. Also, the fact that administration of anti-CTLA-4 mAb can stimulate the immune system in an uncontrolled manner, which can result in the development of autoimmune or lymphoproliferative diseases, could limit the use of such an approach in a clinical setting [15, 40, 41].

In conclusion, our experiments show that in the murine antibody response to caps-PS, blocking CTLA-4 results in a dose-dependent generation of a strong isotype-switched and protective caps-PS-specific antibody response, that CD4(+) as well as CD8(+) T lymphocytes are involved in the CTLA-4-mediated enhancement of the antibody response to caps-PS, and that administration of anti-CTLA-4 results in a markedly increased production of IL-2, IL-4, and IFN-γ by T lymphocytes.

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