CpG and Non-CpG Oligodeoxynucleotides Directly Costimulate Mouse and Human CD4 + T Cells through a TLR9- and MyD88-Independent Mechanism

This information is current as of December 26, 2012.

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CpG and Non-CpG Oligodeoxynucleotides Directly Costimulate Mouse and Human CD4⁺ T Cells through a TLR9- and MyD88-Independent Mechanism

Angela Landrigan, Michael T. Wong, and Paul J. Utz

TLR ligands are known to activate APCs, but direct T cell responsiveness to TLR ligands is controversial. Because of their clinical relevance, we performed in-depth studies of the effects of the TLR9-associated ligands, oligodeoxynucleotides (ODNs), on highly purified T lymphocytes. Both CpG and non-CpG ODNs directly costimulate mouse and human CD4⁺ T cells, resulting in activation marker upregulation, cytokine secretion, elevated TCR phosphorylation, and proliferation. Surprisingly, ODN costimulation occurred independently of TLR9 and MyD88, as well as ICOS, CD28, and TRIF. TLR9-antagonist ODNs likewise promoted T cell activation, which has important implications for the study of these "inhibitory" ODNs in inflammatory diseases. Cytokine profiling revealed that ODNs promote polarization of distinct Th subsets, and that ODNs differentially affect human naive and memory T cells. Our studies reveal a striking and unexpected ability of ODNs to directly activate and polarize T cells, presenting an opportunity to enhance the paradigm for selection of therapeutic ODNs in humans. *The Journal of Immunology*, 2011, 187: 3033–3043.

Toll-like receptor ligands are conserved elements of pathogens that have been extensively studied for their ability to activate APCs, initiating the first line of host defense. Among the motifs that TLRs recognize are bacterial carbohydrates such as LPS, nucleic acids, peptidoglycans, lipoproteins, and peptides such as bacterial flagellin (1, 2). Based on the potent proinflammatory response initiated by unmethylated CpG-containing DNA agonists of TLR9 in APCs, a class of synthetic TLR9 ligands called phosphorothioate oligodeoxynucleotides (ODNs) has been investigated in human clinical trials for allergy, cancer, and autoimmunity (3–13). In mouse models of cancer, CpG-containing ODNs are thought to promote tumor eradication through the TLR9-dependent activation of APCs, resulting in increased uptake and presentation of cancer Ags (14–16). In the autoimmunity setting, TLR9-antagonist ODNs are being investigated for their potential to dampen autoreactivity by inhibiting activation of TLR9 by DNA-containing Ags (17–26). CpG ODNs have been investigated in human clinical trials for treatment of allergic disorders where Ag-conjugated ODNs are thought

to enhance Ag presentation by colocalizing ODNs and Ag to APCs (27). Mouse models have shown that certain CpG sequences deviate the cytokine milieu away from a proallergy Th2 environment (28–31).

In considering approaches to enhance the efficacy of ODNs as therapeutic agents, it is critical to dissect the diverse functions these ligands mediate in a physiological setting. Early characterization of TLRs focused on cells of the innate immune system and APCs; however, studies have emerged demonstrating the presence and function of TLRs on cells formerly not thought to express them, most notably T lymphocytes. Several groups have reported expression of TLRs in T cells, and functional assessments of the costimulatory capacity of TLR ligands when combined with anti-CD3 stimulation have been performed (32–36). The data we present in this study extend these early studies, providing, to our knowledge, the first demonstration of a TLR- and MyD88-independent role for ODNs in the costimulation of T cells. We show that different ODNs promote distinct cytokine secretion profiles from Th cells. These data have important implications for the design of therapeutic ODNs used in clinical trials, as well as for interpretation of ongoing clinical trials. Our studies reveal new insights into the ability of ODNs to activate cell subsets in previously unexplored ways.

Materials and Methods

Mice

BALB/cKa female mice of 6–10 wk age were purchased from the Department of Laboratory Animal Medicine at Stanford University School of Medicine (Stanford, CA). MyD88⁻/⁻ male mice and C57BL/6 controls were a gift from Peggy Ho (L. Steinman Laboratory, Stanford University School of Medicine) and were used at 8 wk age. TLR9⁻/⁻ mice were a gift from Shizuo Akira (Osaka University), obtained directly from Ronald Levy (Stanford University School of Medicine), and female mice were used at 8 wk age. CD28⁻/⁻, ICOS⁻/⁻, and Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF)⁻/⁻ female mice and C57BL/6J controls were purchased from The Jackson Laboratory and used at 8 wk of age. All mice used in this study were maintained under standard conditions at the Stanford University Research Animal Facility. All animal experiments were approved and performed in compliance with the guidelines of the Institutional Animal Care and Use Committee.

Mouse cell isolation and culture

Mouse spleens and lymph nodes were homogenized and filtered using 70-μm nylon cell strainers (BD Biosciences) and a syringe piston. Splenocytes were washed with HBSS for 7 min at 300 × g and gently resuspended in 1 ml PBS plus 2 ml ACK lysis buffer to lyse RBCs for 3 min at 4°C. For R&D/MACS-purified cells (Fig. 2*A*, 2*B*, 2*D*, Supplemental Fig.

2), splenocytes were then applied to CD3-negative selection columns (R&D Systems) followed by CD4-negative selection using magnetic beads (Miltenyi Biotec). Cells were 98% pure by flow cytometry (CD3$^+$CD4$^+$). Cells were kept at 4°C for all steps during isolation. For FACS-purified cells (all other figures), splenocytes were first enriched by CD3-negative selection columns (R&D Systems) and subsequently Fc blocked with anti-mouse CD16/32 (eBioscience; clone 93) and stained with the following
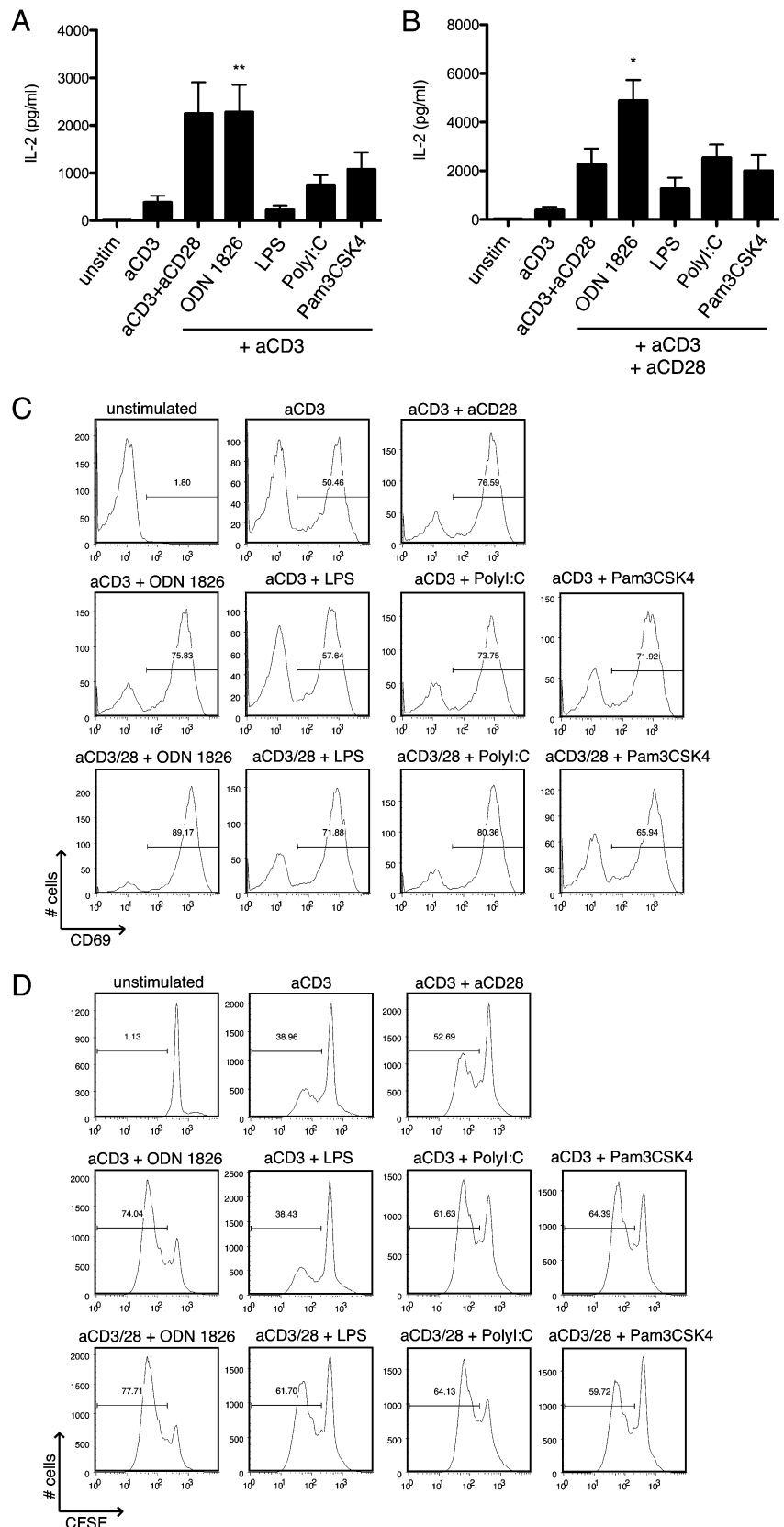
FIGURE 1. TLR ligands costimulate mouse CD4$^+$ T cells, resulting in cytokine secretion, activation marker upregulation, and proliferation. *A* and *B*, FACS-purified (99.9% pure) mouse CD4$^+$ T cells were stimulated with anti-CD3 (5 μg/ml) plus anti-CD28 (5 μg/ml), ODN 1826, LPS, poly (I:C), or Pam3CSK4 as indicated. Supernatants were harvested after 24 h and IL-2 was measured by ELISA. Error bars represent the SEMs of eight independent experiments. **$p = 0.006$, comparing the anti-CD3 and anti-CD3 plus ODN 1826 conditions; *$p = 0.03$, comparing the anti-CD3 plus anti-CD28 and the anti-CD3 plus anti-CD28 plus ODN1826 conditions. Whereas increases were observed with poly(I:C) and Pam3CSK4 costimulation, these conditions had p values >0.05 and therefore those observed increases were not deemed statistically significant. The p values were calculated using an unpaired t test. *C*, T cells were isolated and stimulated as described in *A* and *B*. After 24 h, cells were stained for the early marker of activation CD69 and analyzed by flow cytometry. Data are representative of four independent experiments, with the p value comparing the anti-CD3 and anti-CD3 plus ODN 1826 conditions calculated to be 0.02 using an unpaired t test. *D*, CFSE-labeled T cells were stimulated for 4 d as described in *A* and *B* and subsequently analyzed by flow cytometry to determine the percentage of dividing cells. Data are representative of three independent experiments, with the p value comparing the anti-CD3 and anti-CD3 plus ODN 1826 conditions calculated to be 0.003.

Abs: allophycocyanin-Alexa 750-anti–mouse CD4 (eBioscience; L3T4, clone RM4-5), FITC-anti–mouse CD45R (eBioscience; B220, clone RA3-6B2), FITC-anti–mouse pan-NK (eBioscience; CD49b, clone DX5), FITC-anti–mouse CD11b (eBioscience; clone M1/70), and FITC-anti–mouse CD11c (eBioscience; clone N418). Cells were sorted using a four-way purity mask on either a BD FACSVantage or a BD FACSAria II (BD Biosciences). Cells were cultured at 37°C with 5% CO_2 in flat-bottom 96-well plates (BD Biosciences) in RPMI 1640 (Invitrogen) supplemented with L-glutamine (2 mM; Invitrogen), sodium pyruvate (1 mM; Invitrogen), HEPES (25 mM; Invitrogen), nonessential amino acids (0.1 mM; Invitrogen), penicillin (100 U/ml; Invitrogen), streptomycin (0.1 mg/ml; Invitrogen), 2-ME (5×10^{-5} M; Sigma-Aldrich), and FCS (10%; Omega Scientific). For endosomal inhibition, cells were stimulated in the presence of 25 μM chloroquine for 24 h. Supernatants were harvested and analyzed by ELISA.

Human cell isolation and culture

Enriched CD4^{+} T cells were prepared from leukoreduction system apheresis chambers obtained from healthy donors (Stanford University Blood Center) using a RosetteSep human CD4^{+} T cell enrichment kit (StemCell Technologies) followed by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare). The lymphocyte layer was harvested and treated with ACK lysis buffer at room temperature for 5 min and subsequently washed with cold MACS buffer (PBS plus 2% FCS plus 2 mM EDTA). For the remainder of the isolation, cells were kept at 4°C. Whole, naive, and memory CD4^{+} T cells were purified by FACS sorting on a BD FACSAria II (BD Biosciences). In preparation for FACS sorting, cells were blocked with anti-human Fc receptor (Miltenyi Biotec) and then stained with the following Abs: Alexa 700-anti–human CD4 (BD Biosciences; clone RPA-T4), allophycocyanin-anti–human CD45RA (eBioscience; clone HI100), PE-anti–human CD45RO (eBioscience; clone UCHL1), FITC-anti–human CD14 (eBioscience; clone 61D3), FITC-anti–

human CD16 (eBioscience; clone CB16), FITC-anti–human CD19 (eBioscience; clone HIB19), FITC-anti–human CD36 (eBioscience; clone NL07), FITC-anti–human CD56 (eBioscience; clone MEM188), FITC-anti–human CD123 (eBioscience; clone 6H6), and FITC-anti–human CD235a (eBioscience; clone HIR2). FACS-sorted cells were 99.9% pure as confirmed by flow cytometry. Cells were cultured at 37°C with 5% CO_2 in flat-bottom 96-well plates (BD Biosciences) in RPMI 1640 (Invitrogen) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (25 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5×10^{-5}M), and FCS (10%).

Cell stimulation

Mouse T cells were stimulated at 2×10^6 cells/ml with 5 μg/ml plate-bound anti-mouse CD3 (eBioscience; clone 17A2) and 5 μg/ml anti-mouse CD28 (eBioscience; clone 37.51) where indicated. Human T cells were stimulated at 2×10^6 cells/ml with 10 μg/ml plate-bound anti-human CD3 (eBioscience; clone OKT3 for cytokine and activation marker studies, clone UCHT1 for signaling studies) and 10 μg/ml soluble anti-human CD28 (eBioscience) where indicated. LPS, polyriboinosinic-polyribocytidilic acid [(poly(I:C)], and Pam3CSK4 were purchased from InvivoGen. Phosphorothioate DNA ODNs were synthesized at the Stanford University Protein and Nucleic Acid Facility (Stanford University School of Medicine, Stanford, CA) and have the following sequences: ODN 1668, 5′-TCCATGACGTTCCTGATGCT-3′; ODN 1668 control, 5′-TCCATG-AGCTTCCTGATGCT-3′; ODN 1826, 5′-TCCATGACGTTCCTGAC-GTT-3′; ODN 1826 control, 5′-TCCATGAGCTTCCTGAGCTT-3′; ODN 2006, 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′; ODN 2006 control, 5′-TGCTGCTTTTGTGCTTTTGTGCTT-3′; ODN 2088, 5′-TCCTGGCGG-GGAAGT-3′; ODN 2088 control, 5′-TCCTGAGCTTGAAGT-3′; ODN 2395, 5′-TCGTCGTTTTCGGCGCGCGCCG-3′; ODN 2395 control, 5′-TGCTGCTTTTGGGGGGCCCCCC-3′; G-ODN, 5′-CTCCTATTGGGG-GTTTCCTAT-3′; ODN M362, 5′-TCGTCGTCGTTCGAACGACGTTG-
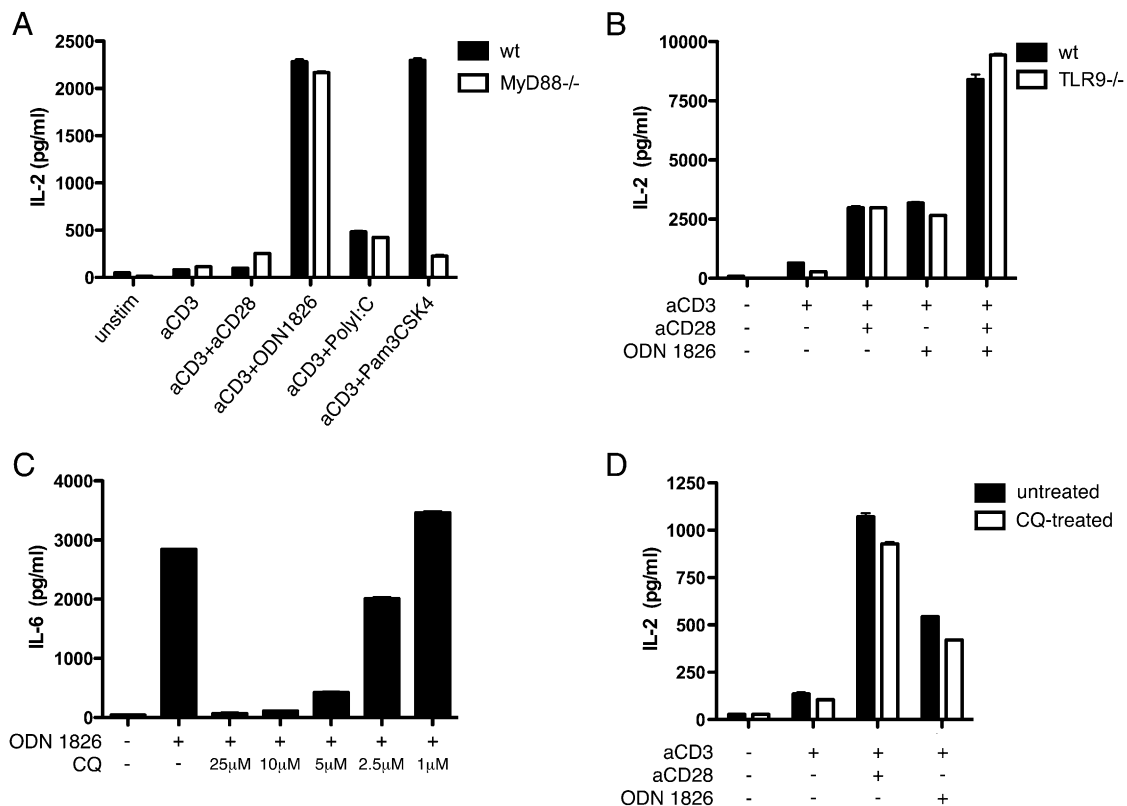
FIGURE 2. ODN 1826 costimulates mouse CD4^{+} T cells independently of traditional TLR signaling mediators. All supernatants were analyzed for the presence of cytokines indicated by ELISA after 24 h stimulation. Error bars represent the SDs of triplicate ELISA wells. Cells were MACS purified (98% CD3^{+}CD4^{+} cells with an additional 1% CD3^{+}CD8^{+} cells). *A*, Wild-type C57BL/6 and MyD88^{−/−} T cells were stimulated with costimulatory TLR ligands. Data are representative of four independent experiments. Note that we consistently observed weak costimulation of C57BL/6 T cells by anti-CD28, resulting in a perceived inflation of ODN 1826 costimulation as compared with anti-CD28 costimulation that is not apparent for BALB/c T cells described elsewhere in this article. *B*, Wild-type BALB/c and TLR9^{−/−} T cells were stimulated with combinations of anti-CD3, anti-CD28, and ODN 1826. Data are representative of five independent experiments. *C*, Splenocytes were incubated with ODN 1826 and titrated concentrations of chloroquine. *D*, T cells were stimulated with combinations of anti-CD3, anti-CD28, and ODN 1826 in the presence or absence of chloroquine. Chloroquine concentration was determined in C. Data are representative of six independent experiments.

AT-3′; ODN M362 control, 5′-TGCTGCTGCTTGCAAGCAGCTTGAT-3′. Results with ODN 1826 were confirmed with reagent purchased from InvivoGen. Mixed phosphorothioate-phosphodiester ODNs were purchased from Eurofins MWG Operon and have the following sequences (phosphorothioate regions are in lowercase letters, phosphodiester regions are in capital letters): ODN 1585, 5′-ggGGTCAACGTTGAgggggg-3′; ODN 1585 control, 5′-ggGGTCAAGCTTGAgggggg-3′; ODN 2216, 5′-ggGGGACGATCGTCgggggg-3′; ODN 2236, 5′-gggGACGACGTCGTGggggg-3′.

Analysis of cytokine secretion

Cell supernatants were harvested and analyzed for IL-2, IL-6, IL-9, and IL-12p40 secretion by ELISA using BD OptEIA ELISA sets (BD Biosciences) and for IL-4, IL-17A, and IFN-γ by ELISA using eBioscience Ab pairs in 96-well Nunc MaxiSorp plates (Nalgene Nunc). Tetramethylbenzidine substrate (Dako) was used in the developing step, and OD values were determined at 450 nm. Absorbance values from blank wells were subtracted. Data were plotted using GraphPad Prism software.

Intracellular phospho-flow cytometry

MACS-purified human CD4+ T cells (99% pure) were stimulated as described above. Cells were fixed and permeabilized using eBioscience fixation and permeabilization buffers. Permeabilized cells were separated into two staining panels and stained with combinations of Alexa 488-anti–human SLP76 (BD Biosciences; clone J141-668.36.58), PE-anti–human p-CD247 (BD Biosciences; clone K25-407.69), PE-anti–human phosphorylated phospholipase C (PLC)γ2 (BD Biosciences; clone K86-689.37; cross-reactive with p-PLCγ1 in T cells), PerCP-eFluor 710-anti–human CD45RO (eBioscience; clone UCHL1), and PE-Cy7-anti–human CD45RA (eBioscience; clone HI100). Stained cells were analyzed by flow cytometry on a BD LSR II (BD Biosciences). Data were analyzed using Cytobank (Menlo Park, CA).

Proliferation assay

Purified cells were washed twice with cold serum-free RPMI 1640 and labeled with 0.42 μM CFSE for 15 min at 37°C. Cells were washed twice with cold serum-free RPMI 1640, resuspended in complete media, and stimulated for 4 d as described above. On day 4, cells were harvested, washed with cold PBS, and analyzed for CFSE fluorescence by flow cytometry using a FACScan (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Activation marker upregulation

Stimulated cells were harvested, washed with FACS buffer containing PBS (Invitrogen), 2% FCS (Omega Scientific), and 0.02% NaN₃ (Sigma-Aldrich). Cells were stained with PE-anti–mouse CD69 (eBioscience; clone H1.2F3) or PE-Cy5-anti–human CD25 (eBioscience; clone BC96) for 30 min on ice in the dark. Cells were washed with FACS buffer and analyzed by flow cytometry using a BD FACScan (BD Biosciences). Data were analyzed using FlowJo Software (Tree Star).

Results

TLR ligands costimulate highly purified mouse CD4+ T cells

To test the hypothesis that TLR ligands directly costimulate CD4+ T cells, we studied highly purified T cells to exclude the possibility of crosstalk from APCs. We FACS sorted mouse CD4+ T cells to 99.9% purity and stimulated them with anti-CD3 in the presence of anti-CD28 or TLR ligands as costimulatory factors. All TLR ligands were titrated and the concentration resulting in maximal IL-2 secretion was used. ODN 1826 (a TLR9 agonist) costimulated T cells, resulting in IL-2 secretion after 24 h, with a statistically significant *p* value of 0.006 as compared with anti-CD3 stimulation alone. Costimulation by poly(I:C) (a TLR3 agonist) and Pam3CSK4 (a TLR2 agonist) resulted in increased IL-2 secretion that was not deemed statistically significant, and costimulation by LPS did not result in altered IL-2 secretion (Fig. 1A). Similarly, ODN 1826 augmented stimulation with anti-CD3 plus anti-CD28 with a statistically significant *p* value of 0.03 as compared with costimulation with anti-CD28 alone, whereas addition of Pam3CSK4, poly(I:C), or LPS to anti-CD3 plus anti-CD28 did not alter IL-2 production in a statistically significant

manner (Fig. 1B). TLR ligands alone lacked the capacity to promote IL-2 secretion at any concentration or time point tested (unpublished data).

Costimulation or augmentation of anti-CD3 plus anti-CD28 stimulation with either ODN 1826, poly(I:C), or Pam3CSK4 resulted in the upregulation of the early activation marker CD69 to levels proportional to IL-2 output; however, as seen with IL-2 production, only costimulation by ODN 1826 showed increases that were statistically significant (*p* = 0.02; Fig. 1C). In contrast to the IL-2 data, augmentation of anti-CD3 plus anti-CD28 stimulation of T cells with ODN 1826 did not additionally alter CD69 expression in a statistically significant manner. TLR ligands alone did not augment CD69 expression (unpublished data).

We tested the hypothesis that TLR ligand costimulation alters T cell proliferation. Costimulation with ODN 1826, poly(I:C), or Pam3CSK4 resulted in 10–20% greater proliferation than did costimulation with anti-CD28 (Fig. 1D); however, only costimulation with ODN 1826 resulted in statistically significant increases in proliferation (*p* = 0.003). As seen with CD69 expression, addition of ODN 1826 to anti-CD3 plus anti-CD28–stimulated T cells appeared to augment proliferation, although this difference was not statistically significant. TLR ligands alone had no effect on T cell proliferation (unpublished data).

TLR ligands costimulate mouse CD4+ T cells in a TLR9-, MyD88-, and endosome-independent manner

The ligands for TLR2 and TLR9 signal through the intracellular protein MyD88 in APCs. We tested the hypothesis that these ligands likewise signal through MyD88 in CD4+ T cells. Using T cells from mice lacking the MyD88 protein and IL-2 secretion as the readout (Fig. 2A), we determined that Pam3CSK4 indeed signals through MyD88. Surprisingly, ODN 1826 costimulated T cells independently of MyD88 (Fig. 2A). TLR3 ligand poly(I:C) signals through TRIF and not MyD88 in APCs, and our studies likewise showed that poly(I:C) costimulation of CD4+ T cells occurs independently of MyD88 (Fig. 2A). These studies also showed that the costimulatory effects of TLR ligands on CD4+ T cells are not limited to the BALB/c strain, as mice used in these experiments were on the C57BL/6 background. Note that we consistently observed weak costimulation of C57BL/6 T cells by anti-CD28, resulting in a greater proportionate level of ODN
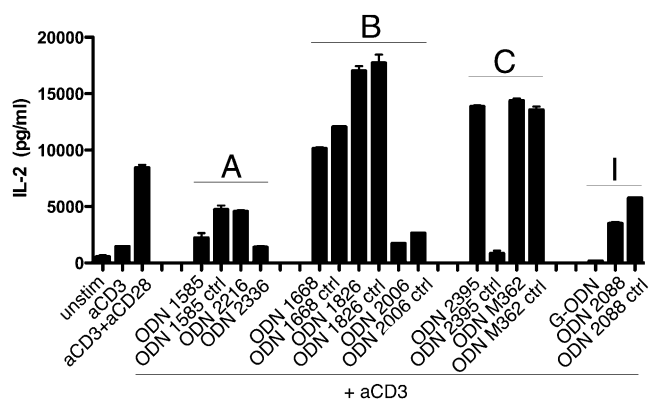
FIGURE 3. CpG, non-CpG, and TLR9 antagonist ODNs costimulate mouse CD4+ T cells. FACS-purified (99.9% pure) T cells were stimulated with anti-CD3 plus anti-CD28 or a panel of ODNs for 24 h. A, B, C, and I denote class A, class B, class C, and TLR9-antagonist ODNs, respectively, based on the classification system of TLR9 function in APCs. The ODN suffix "ctrl" indicates a non-CpG control for the corresponding CpG-containing ODN. Supernatants were assessed for the presence of IL-2 after 24 h. Error bars represent the SDs of triplicate ELISA wells. Data are representative of six independent experiments.

1826 costimulation as compared with anti-CD28 costimulation of C57BL/6 T lymphocytes that is not apparent for BALB/c T cells described elsewhere in this article.

Because of potent costimulatory effects, lack of cell toxicity, ease of synthesizing sequence variants, and implications for ongoing human clinical trials using synthetic ODNs, we focused our efforts on further elucidating the mechanisms behind ODN 1826 costimulation of CD4+ T cells. Titration of ODN 1826 revealed that costimulation increases within a concentration range of 0.6–20 μM (Supplemental Fig. 1). Using the concentration that promoted maximal IL-2 secretion, we tested the hypothesis that costimulation occurs through TLR9 via a MyD88-independent mechanism. Using TLR9 knockout mice and IL-2 secretion as a readout, we determined that ODN 1826-mediated costimulation does not require TLR9 (Fig. 2B). To determine whether endosomal acidification was required for costimulation, we treated cells with chloroquine at a concentration shown to inhibit TLR9-dependent IL-6 secretion by splenocyte-derived APCs (Fig. 2C). We confirmed our results using a second inhibitor of endosomal acidification, bafilomycin A1, which also does not inhibit ODN 1826-mediated costimulation (data not shown). We determined that traditional costimulation with anti-CD28 does not require acidified endosomes (Fig. 2D). We likewise found that costimu-lation does not occur via the TLR3-associated signaling mediator TRIF (Supplemental Fig. 2) or through two well-described co-stimulatory receptors on T cells, ICOS and CD28 (Supplemental Fig. 3). These data reveal the presence of a novel, previously undiscovered signaling pathway for ODN costimulation in T cells.

CpG, non-CpG, and TLR9-antagonist ODNs costimulate mouse CD4+ T cells

To further characterize ODN effects on T cells, we tested the costimulatory ability of a panel of ODNs that vary in class and nucleotide sequence. Conventional ODN classes are defined by their TLR9-associated APC cell subsets. In our studies, class B and class C ODNs most potently costimulated T cells (Fig. 3); however, not all ODNs within these classes had the capacity to costimulate, as measured by IL-2 secretion, suggesting that our findings are not the result of nonspecific charge interactions. We further observed that ODNs lacking CpG motifs (labeled as control) potently cos-timulated T cells. CpG motifs have been shown to be essential for TLR9-mediated signaling in APCs induced by phosphorothioate (37). To our surprise, TLR9-antagonist ODNs that inhibit APC activation also costimulated T cells. Using genetic knockout mice lacking TLR9, we found that all ODNs costimulated T cells in-dependently of TLR9 (unpublished data), further supporting our
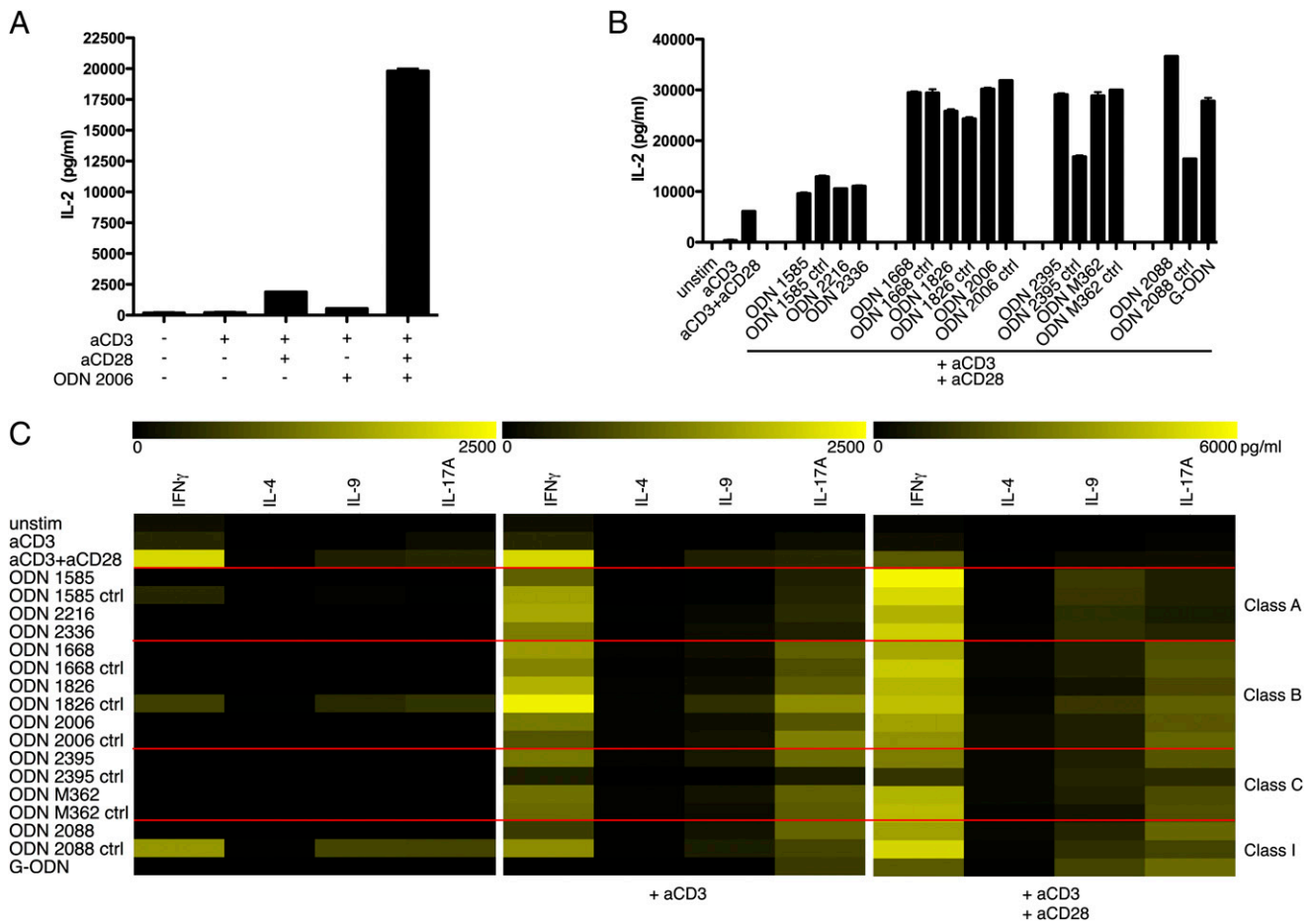
FIGURE 4. Human CD4+ T cell activation is potently augmented by ODN costimulation, resulting in distinct cytokine profiles. *A,* FACS-purified (99.9% pure) T cells isolated from healthy donor blood were stimulated with combinations of anti-CD3, anti-CD28, and ODN 2006 for 48 h. Supernatants were collected and assayed by ELISA for IL-2. Error bars represent the SDs of triplicate ELISA wells. Results are representative of 14 independent experiments from 14 different donors. *B* and *C,* T cells were stimulated with anti-CD3, anti-CD28, and a panel of ODNs, arranged by class in the rows. Class I denotes TLR9-antagonist ODNs and a control. The ODN suffix "ctrl" indicates a non-CpG control ODN for the CpG-containing ODNs. After 48 h, supernatants were assessed by ELISA for the presence of IL-2, IFN-γ, IL-4, IL-9, and IL-17A. The *left panel* depicts data from T cells stimulated with ODN alone; the *middle panel* depicts results from T cells stimulated with anti-CD3 plus ODN; and the *right panel* shows data from T cells stimulated with anti-CD3, anti-CD28, and ODN. Results are representative of seven independent experiments from seven different healthy donors.

conclusion that ODN-driven costimulation of CD4^{+} T cells does not occur through TLR9.

CpG, non-CpG, and TLR9-antagonist ODNs enhance activation of human T cells

To translate mouse findings to humans, we tested the hypothesis that ODNs costimulate FACS-purified human CD4^{+} T cells. We began by testing the ability of ODN 2006, which is currently in human cancer clinical trials (9, 11–13), to costimulate T cells. Whereas ODN 2006 alone costimulated T cells weakly, it greatly augmented anti-CD3 plus anti-CD28 stimulation, with a range of 3- to 23-fold increase in IL-2 secretion as compared with anti-CD3 plus anti-CD28 stimulation, as assessed across 14 healthy donors (mean augmentation, 9-fold) (Fig. 4A). Titration of ODN 2006 revealed that activity peaked at 20 μM, but was present at concentrations as low as 0.63 μM, the lowest concentration tested (Supplemental Fig. 4).

We determined the profile of ODNs that augment human CD4^{+} T cell activation. Similar to findings in mouse T cells, we found that both CpG and non-CpG ODNs augmented human CD4^{+} T cell activation (Fig. 4B). Likewise, class B and class C ODNs most potently stimulated human T cells to produce IL-2. ODNs designated as antagonistic to TLR9 function potently augmented human T cell stimulation. Taken together, these data suggest a TLR9-independent mechanism for ODN-mediated augmentation of human CD4^{+} T cell activation.

ODNs promote distinct Th cytokine profiles from human T cells

We next determined the cytokine profile of human CD4^{+} T cells stimulated with ODN. We stimulated FACS-purified whole CD4^{+} T cells with anti-CD3 plus anti-CD28 plus a panel of ODNs and measured the concentration of IFN-γ (Th1 cytokine), IL-4 (Th2 cytokine), IL-9 (which defines Th9 cells), and IL-17A (secreted by Th17 cells) in the supernatant after 48 h. Interestingly and quite unexpectedly, we observed that different ODNs promoted distinct patterns of cytokine secretion (Fig. 4C). For example, while ODN 2006 control and ODN 2088 costimulation favored IL-17A secretion over IFN-γ secretion, ODN 1826 control and ODN 2216 promoted the opposite profile (Fig. 4C). We also noted class-wide differences: whereas class A ODNs promoted little secretion of IL-2 and IL-17A and instead favored IL-9 secretion, class B and

class C ODNs promoted high levels of IL-2 and IL-17A and lower levels of IL-9. Because naive T cells stimulated with anti-CD3, anti-CD28, and ODN do not secrete cytokines at day 2 or day 4 (unpublished data), we think that treatment with ODNs differentially promotes the expansion of pre-existing polarized subsets of whole CD4^{+} T cells, resulting in a distinct cytokine milieu for each ODN.

We analyzed how the cytokine environment changes over time following stimulation with ODN 2006. Human CD4^{+} T cells were stimulated with combinations of anti-CD3, anti-CD28, and ODN 2006. Day 2 and day 4 supernatants were assessed for the presence of IL-2, IFN-γ, IL-4, IL-9, and IL-17A (Fig. 5). Notably, the presence of ODN 2006 prevented the increase in IFN-γ and IL-17A observed at day 4 in anti-CD3– plus anti-CD28–stimulated cells. ODN 2006 prevented both the day 2 and day 4 secretion of IL-9. Exposure to ODN 2006 was associated with increased levels of IL-4 at day 4 as compared with stimulation with anti-CD3 and anti-CD28 alone. Therefore, whereas ODN 2006 promoted an early Th1 response, it deviated cytokine secretion toward a Th2 response by day 4.

Given the ability of different ODNs in the panel to promote diverse cytokine outcomes at day 2 (Fig. 4), we hypothesized that different ODNs might likewise promote distinct changes in cytokine secretion by day 4 (Fig. 6). We stimulated FACS-purified human CD4^{+} T cells with combinations of anti-CD3, anti-CD28, and a panel of ODNs and measured the concentrations of IL-2, IFN-γ, IL-4, IL-9, and IL-17A at day 4. Cells stimulated with anti-CD3, anti-CD28, and ODN 2006 produced lower levels of IFN-γ and higher levels of IL-4 at day 4 as compared with cells stimulated with anti-CD3 plus anti-CD28 (Fig. 6). Several other ODNs had similar effects. In contrast, ODN 1826 and ODN 1585control promoted high levels of IFN-γ secretion and low levels of IL-4 when combined with anti-CD3 plus anti-CD28 stimulation. Overall, we conclude that different ODNs promote distinct day 4 cytokine profiles.

ODN differentially augments human memory Th cell activation

Given that human CD4^{+} T cells purified from peripheral blood exist as a heterogeneous population, we compared naive and memory cell responsiveness to ODNs. FACS-purified CD4^{+} CD45RO^{+}CD45RA^{−} memory and CD4^{+}CD45RO^{−}CD45RA^{+} na-
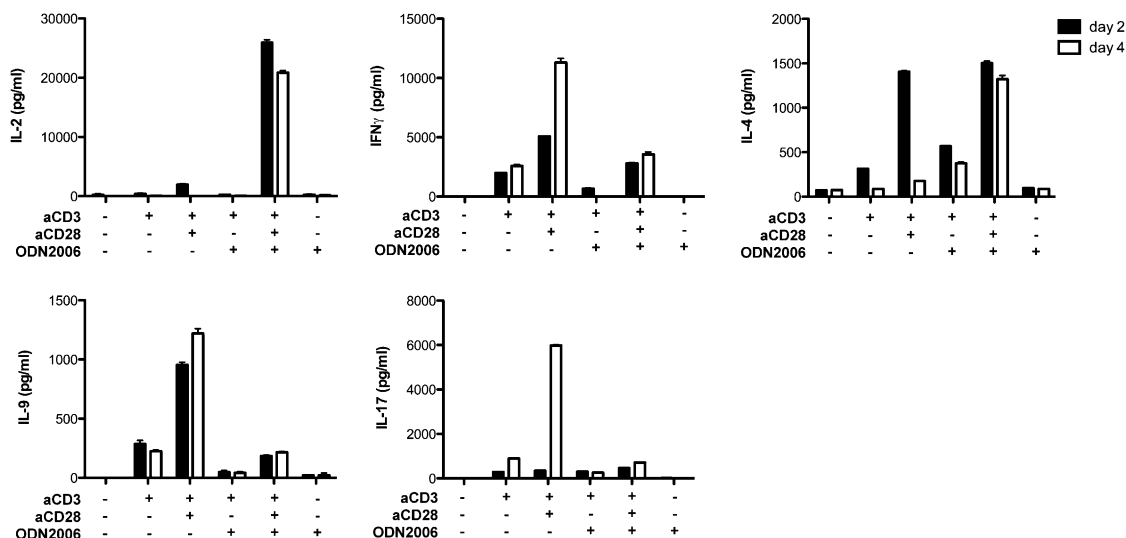
FIGURE 5. ODN 2006 costimulation alters the nature and kinetics of human CD4^{+} T cell stimulation. FACS purified (99.9% pure) T cells were stimulated with anti-CD3, anti-CD28, and ODN 2006 as indicated for 2 d or 4 d. Supernatants were assessed for IL-2, IFN-γ, IL-4, IL-9, and IL-17A by ELISA. Error bars represent the standard deviations of triplicate ELISA wells. Data are representative of three independent experiments from three healthy donors.
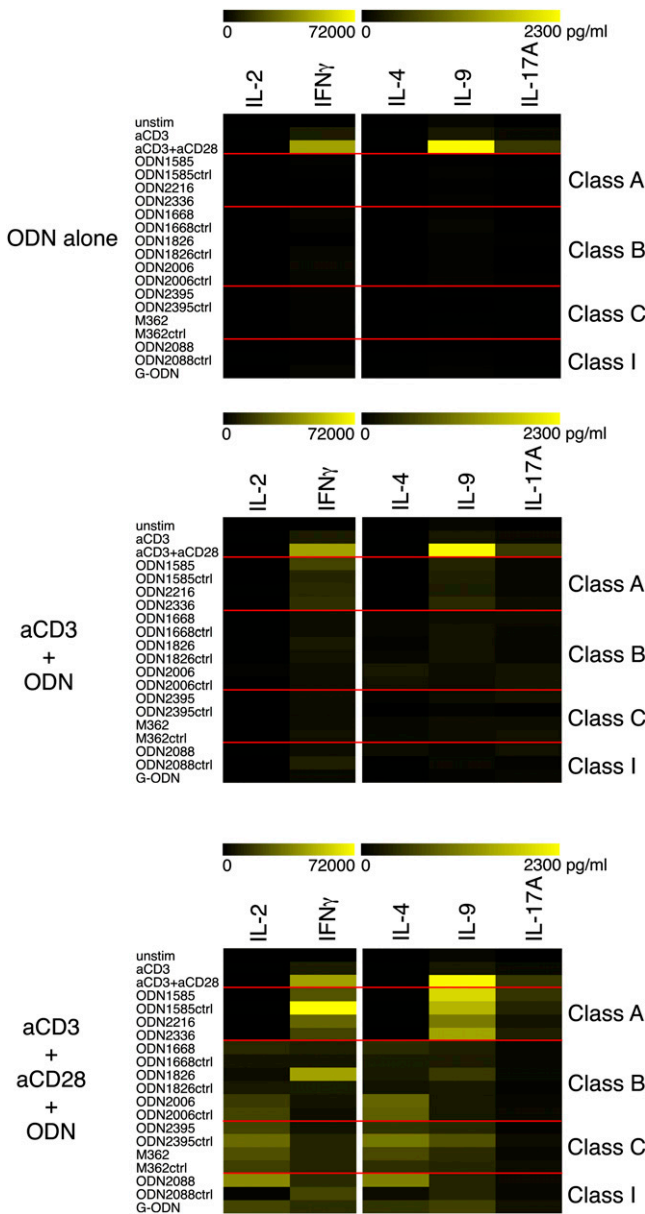
FIGURE 6. ODNs differentially regulate cytokine secretion by day 4 of stimulation. FACS-purified (99.9% pure) human CD4$^+$ T cells were stimulated for 4 d with combinations of anti-CD3, anti-CD28, and a panel of ODNs, arranged by class in the rows. Class I denotes TLR9-antagonist ODNs and a control. The ODN suffix "ctrl" indicates a non-CpG control for the corresponding CpG-containing ODN. IL-2, IFN-γ, IL-4, IL-9, and IL-17A in cell supernatants were assayed by ELISA and are displayed in the columns. Results are representative of six independent experiments from six different healthy donors.

ive T cells were stimulated with combinations of anti-CD3, anti-CD28, and ODN 2006. The secretion of IL-2 by memory T cells stimulated with anti-CD3 plus anti-CD28 for 48 h was profoundly augmented by the addition of ODN 2006 (Supplemental Fig. 5A). Among six healthy human donors tested, five donors showed an increase of 3- to 37-fold, comparing anti-CD3 plus anti-CD28 stimulation to a combination of anti-CD3, anti-CD28, and ODN 2006. The sixth donor showed an even more striking 172-fold increase in IL-2 secretion. In contrast to the response in memory cells, ODN 2006 inhibited IL-2 secretion (Supplemental Fig. 5A) and CD25 upregulation (Supplemental Fig. 5B) by naive T cells after 48 h, without compromising cell viability (unpublished data). By day 4, IL-2 secretion and CD25 upregulation were comparable

between anti-CD3 plus anti-CD28 conditions with or without ODN 2006 (unpublished data). These data reveal the important and surprising finding that ODN 2006 differentially affects human memory and naive T cell activation.

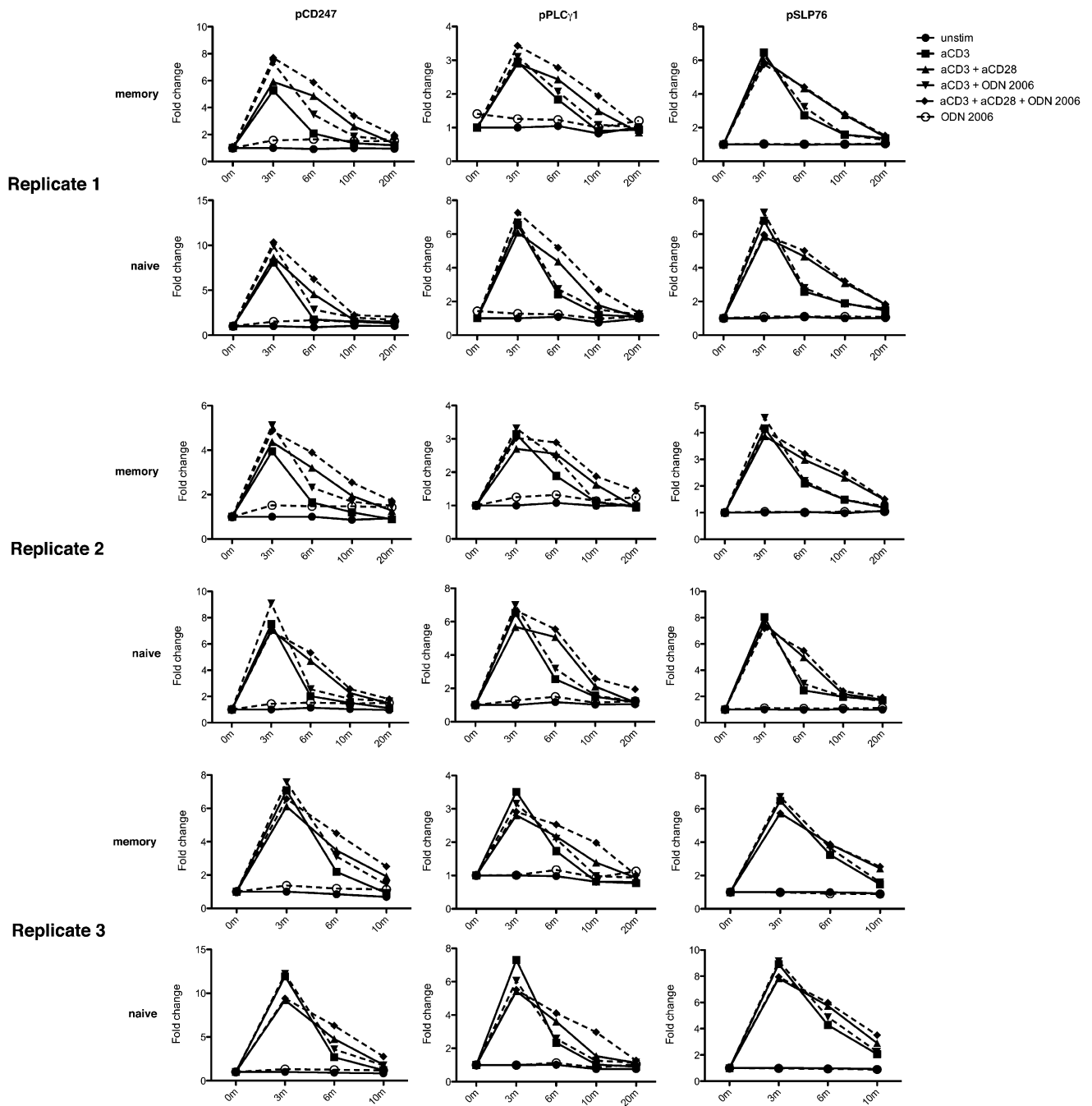### ODN induces elevated phosphorylation of TCR and TCR-proximal proteins

Mechanistic signaling studies were undertaken to determine whether the presence of ODN alters the level or kinetics of phosphorylation of TCR and TCR-proximal proteins. Human CD4$^+$ T cells from peripheral blood were purified to 99% purity and stimulated with combinations of anti-CD3, anti-CD28, and ODN 2006 for intervals up to 20 min. Stimulated cells were fixed, permeabilized, and stained with Abs to p-CD247, p-PLCγ1, and p-SLP76. Cells stimulated with anti-CD3 plus ODN 2006 or anti-CD3 plus anti-CD28 plus ODN 2006 showed an increase in CD247 (TCRζ) peak phosphorylation at 3 min in both CD4$^+$CD45RA$^+$CD45RO$^-$ naive and CD4$^+$CD45RO$^+$CD45RA$^-$ memory T cells as compared with anti-CD3 or anti-CD3 plus anti-CD28 alone (Fig. 7). This increased level of phosphorylation was sustained throughout the time course until the 20 min time point. The presence of ODN 2006 likewise resulted in an increase in peak phosphorylation of the TCR-proximal signaling protein PLCγ1, which was likewise sustained until the 20 min time point. In contrast, no changes in the phosphorylation status of SLP76 were observed at any time point. Overall, these results demonstrate that ODN 2006 induces hyperphosphorylation of the TCRζ-chain and propagates a signal through a pathway involving PLCγ1 but not SLP76.

Discussion

The first responders to host invasion are often the cells of the innate immune system, such as macrophage and dendritic cells, which express highly conserved pattern recognition receptors such as TLRs that detect conserved motifs associated with invading pathogens and cellular stress (1, 38–40). Phosphorothioate-modified ODNs are mimics of natural DNA motifs associated with pathogens, and are often employed in culture and clinical applications due to their enhanced stability and potency in modulating the inflammatory response. In B cells and cells of the innate immune system, it is traditionally thought that phosphorothioate-modified ODNs propagate signals through endosome-expressed TLR9 and the associated MyD88 signaling protein, resulting in an inflammatory response (41, 42).

Although previous studies of TLR function in T cells have revealed the ability of TLR ligands to induce an inflammatory response in T cells, these studies assumed that TLR ligands acted on T cells through MyD88 and TLRs because of the role of these proteins in innate immune signaling, without directly testing this hypothesis. We now provide critical mechanistic insights into this observation using mouse genetic knockout models to test this hypothesis. To our knowledge, we provide the first conclusive evidence that ODNs costimulate CD4$^+$ T cells via a TLR9- and MyD88-independent mechanism. We demonstrate that this costimulation does not require two well-described costimulatory receptors, CD28 and ICOS. We report further characterization of the receptor in showing that costimulation does not require acidified endosomes and that both CpG- and non-CpG–containing ODNs are capable of costimulating CD4$^+$ T cells, further confirming our studies in T cells derived from TLR9 and MyD88 knockout mice. It remains possible that other known costimulatory molecules are involved, and their identification is an area of active investigation. To our knowledge, our data provide the first insights into a novel pathway by which T cells are observed to function beyond their traditional roles in adaptive immunity.

FIGURE 7. ODN 2006 induces hyperphosphorylation of TCR and TCR-proximal signaling proteins. MACS-purified (99% pure) human CD4$^+$ T cells were stimulated for 0, 3, 6, 10, or 20 min with combinations of anti-CD3, anti-CD28, and ODN 2006 as indicated. All conditions contain 4 μg/ml goat anti-mouse crosslinking Ab. Cells were fixed, permeabilized, and stained with Abs against p-CD247 (TCRζ), p-PLCγ1, and p-SLP76. Replicates from three healthy donors are shown.

Understanding the mechanism by which T cells recognize conserved motifs holds great importance for harnessing the power of this new function of T cells to transform the field of therapeutic ODNs.

Although our studies represent, to our knowledge, the first mechanistic studies in CD4$^+$ T cells, recent studies have emerged demonstrating TLR9- and MyD88-independent mechanisms for ODN stimulation of non-T cells (43–48). Using genetic knockout mice, these studies have demonstrated the ability of ODNs to induce activation marker upregulation, cytokine secretion, and proliferation. Studies by Bendigs et al. (36) and Lipford et al. (49) have also suggested ODNs may possess the ability to activate mouse CD8$^+$ T cells through a TLR9- and MyD88-independent

mechanism. These studies provide precedent in non-CD4$^+$ T cell systems for our studies, which provide direct genetic evidence of TLR9- and MyD88-independent signaling in CD4$^+$ T cells using knockout mice lacking TLR9 and MyD88.

Recently, Lancioni et al. (50) shed light on an important underlying factor in interpreting sometimes conflicting reports of the ability of TLR ligands to costimulate T cells directly—that of T cell culture purity. We have made a point of confirming our conclusions of T cell responsiveness to TLR ligands using cells isolated by FACS to 99.9% purity.

Several cytosolic DNA sensors that promote an immune response in a TLR-independent manner in APCs have recently been described. Takaoka et al. (51) discovered that the 40-kDa cyto-

solic protein DAI (DLM-1/ZBP1) binds double-stranded DNA, resulting in an IFN response through a mechanism involving TANK-binding kinase-1. There have been several recent reports on the involvement of the novel 39-kDa DNA-binding protein AIM2 in promoting cell death through the activation of IL-1β and caspase-1, in an apoptosis-associated speck-like protein-dependent manner (52–54). Several signaling proteins involved in mediating the response to DNA have also been described (55–60). Of interest given our findings of costimulatory activity of non-CpG ODNs, the stimulator of IFN genes protein that associates with TANK-binding kinase-1 promotes the proinflammatory response of dendritic cells, macrophages, and fibroblasts to a variety of DNA stimuli, including DNA lacking CpG motifs (61, 62). These studies reveal that partially overlapping mechanisms are involved in DNA sensing and the ensuing immune response, and they provide evidence suggesting the existence of unidentified DNA sensors. Whether the ODNs in this study exert their functions on T cells through one of these sensors remains to be elucidated. Phosphorothioate ODNs used clinically do not occur in nature, and it remains possible that sensing and propagation of a functional response may occur by an alternative mechanism.

ODNs are attractive candidates for in-depth study given their ongoing investigation as therapeutic agents in the treatment of allergy, cancer, and autoimmunity, where they have demonstrated favorable safety profiles and tolerability in patients (3–9, 11–13, 27, 63, 64). "Inhibitory" ODNs are thought to bind to and block stimulation through TLR9 or other TLRs in APCs, decreasing the presentation of self-Ag and preventing autoreactivity. Although inhibitory ODNs may block TLR9 in APCs, our data indicate their potential to directly activate T cells in previously unanticipated ways. Our discovery that non-CpG and TLR9-antagonist ODNs promote distinct T cell cytokine secretion outcomes may be leveraged in a beneficial way in treating autoimmunity. For example, ODNs that either fail to signal through or actively inhibit TLR9 in APCs could be investigated for their potential to induce cytokine profiles that ameliorate disease in patients.

Favorable anti-cancer responses are traditionally associated with a Th1 cytokine profile, and recent evidence has suggested that Th17 cells may facilitate inflammatory responses (65). Using a panel of ODNs, we identified ODNs that directly promote Th1 and Th17 responses from T cells. We hypothesize that the selection of ODNs that promote strong Th1 and Th17 cytokine responses from T cells whereas still retaining the ability to stimulate through TLR9 in APCs may result in enhanced anti-cancer activity of ODNs used clinically. We found two ODNs that approach this ideal in our limited screen. Of the ODNs capable of stimulating human APCs through TLR9, ODN 2216 and ODN 2336 showed enhanced ability to promote IFN-γ secretion without IL-4 secretion when combined with anti-CD3 plus anti-CD28 stimulation, as compared with the effects of ODN 2006 (Fig. 6). It is straightforward to synthesize sequence variants to screen for the ability to not only activate APCs through TLR9, but also to elicit a strong Th1 response directly from T cells. Additionally, costimulation with ODNs is an attractive alternative to CD28 costimulation with respect to patient therapy, as anti-CD28 Abs have demonstrated life-threatening clinical toxicity (66).

In the APC model, ODNs are divided into five classes (class A, class B, class C, control, and inhibitory) based on structural elements. Class A ODNs possess a phosphorothioate backbone region that is flanked by phosphodiester bases and contain polyguanine acid (polyG) tails and palindrome sequences. Class B ODNs are comprised solely of a phosphorothioate backbone and lack polyG tails and palindromes. Class C ODNs contain a phosphorothioate backbone and harbor palindrome sequences, but lack a polyG tail.

Control ODNs lack a CpG motif and the polyG tail and do not activate APCs. Inhibitory ODNs likewise do not have a CpG motif, but contain a polyG string after a C or A base; these ODNs inhibit APC activation through TLR9 by CpG-containing class A, B, and C ODNs. Using this classification system, we have observed trends in our studies of human CD4+ T cells, particularly as related to the class A ODNs. We have observed that class A ODNs promote significantly lower IL-2 secretion and higher IL-9 secretion than do other classes after 24 h stimulation (Fig. 4B, 4C). By day 4, this ability of the class A ODNs to promote IL-9 secretion is profoundly enhanced (Fig. 6, *lower panel*). Class A ODNs also promote higher levels of IL-17A and IFN-γ secretion by day 4, whereas IL-4 and IL-2 levels are dramatically decreased as compared with the other classes (Fig. 6, *lower panel*).

Mechanistically, our data reveal that the addition of ODN 2006 to anti-CD3 plus anti-CD28–stimulated human Th cells results in increased peak phosphorylation of the TCRζ-chain, to a greater extent than is observed with anti-CD28 costimulation alone. Furthermore, ODN 2006 appears to propagate its signal through a pathway involving the TCR-proximal signaling protein PLCγ1 but not SLP76. The mechanism by which distinct ODNs differentially promote Th cytokine profiles remains to be elucidated; however, there is precedent for differential effects of ODNs in studies performed on APCs. For example, recent studies showed that class A and class B ODNs promote distinct cytokine profiles from B cells through localization to distinct intracellular compartments (67). Whether ODNs differentially localize to cell surfaces or within particular intracellular organelles in T cells remains to be studied.

The existence of multiple roles for ODNs in diverse populations of immune cells underscores the need for a more detailed understanding of the physiological role of ODNs. Our data reveal a previously undescribed pathway by which ODNs potently stimulate CD4+ T cells to produce specific cytokine polarization outcomes, particularly in humans. These data bring forth an opportunity to inform the selection of ODNs used in therapeutic settings, taking into account their newfound ability to influence the cytokine environment directly through CD4+ T cell activation.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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