

A Human CD4 Monoclonal Antibody for the Treatment of T-Cell Lymphoma Combines Inhibition of T-Cell Signaling by a Dual Mechanism with Potent Fc-Dependent Effector Activity

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Abstract

Zanolimumab is a human IgG1 antibody against CD4, which is in clinical development for the treatment of cutaneous and nodal T-cell lymphomas. Here, we report on its mechanisms of action. Zanolimumab was found to inhibit CD4⁺ T cells by combining signaling inhibition with the induction of Fc-dependent effector mechanisms. First, T-cell receptor (TCR) signal transduction is inhibited by zanolimumab through a fast, dual mechanism, which is activated within minutes. Ligation of CD4 by zanolimumab effectively inhibits early TCR signaling events but, interestingly, activates signaling through the CD4-associated tyrosine kinase p56^{lck}. An uncoupling of p56^{lck} from the TCR by anti-CD4 allows the kinase to transmit direct inhibitory signals via the inhibitory adaptor molecules Dok-1 and SHIP-1. Second, CD4⁺ T cells are killed by induction of antibody-dependent cell-mediated cytotoxicity, to which CD45RO⁺ cells are more sensitive than CD45RA⁺ cells. Finally, zanolimumab induces down-modulation of CD4 from cell surfaces via a slow Fc-dependent mechanism. In conclusion, zanolimumab rapidly inhibits T-cell signaling via a dual mechanism of action combined with potent Fc-dependent lysis of CD4⁺ T cells and may act long-term by down-regulating CD4. [Cancer Res 2007;67(20):9945–53]

Introduction

The therapeutic activity of CD4 monoclonal antibodies (mAb) in reversal of graft rejection (1), inhibition of various autoimmune disease animal models (2, 3), and human inflammatory diseases, such as rheumatoid arthritis (4, 5), as well as the induction of tolerance (6) and inhibition of HIV-1 infection (7), has been studied extensively over the past 15 years. Despite initial promise, CD4 mAb has only met limited success in the clinical setting, in part because immunogenicity, observed in clinical studies using mouse and chimeric mAb, prevents repeat dosing (8, 9).

Recent clinical progress has, however, been made with zanolimumab, a human antibody against CD4 isolated from

human antibody transgenic mice (10, 11). Zanolimumab was assessed in clinical trials in inflammatory diseases, including rheumatoid arthritis and psoriasis, in which it induced significant T-cell depletion in repeat dosing, and was found safe and well tolerated (12). This profile led to the notion that zanolimumab treatment might provide benefit in T-cell malignancies, and the mAb was entered into clinical trials for the treatment of cutaneous T-cell lymphoma (CTCL; ref. 13) and nodal T-cell lymphoma (14). The mechanisms responsible for decreasing the number of circulating CD4⁺ T cells for CD4 mAb in general, and zanolimumab in particular, have not been elucidated. Possible nonexclusive mechanisms include the compartmentalization of CD4⁺ T cells; induction of Fc receptor (FcR)-mediated effector functions, such as complement-mediated cytotoxicity (CDC; ref. 15) and antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 16); CD4 receptor down-modulation (16–19); inhibition of T-cell activation (16, 18); and induction of apoptosis (20–22).

The inhibitory effects of CD4 mAb on T-cell activation have received much attention. It has long been known that binding of CD4 in the absence of T-cell receptor (TCR) ligation results in decreased TCR-stimulated proliferation, cytokine production, and activation marker expression (23, 24). The cytoplasmic tail of CD4 is associated noncovalently with the p56^{lck} tyrosine kinase (25) and during T-cell activation through the CD4/TCR complex, the juxtaposition of CD4/p56^{lck} and the TCR initiates the TCR signaling cascade. p56^{lck} phosphorylates immunoreceptor tyrosine-based activation motifs located in the CD3 ζ and CD3 ϵ chains (26, 27), resulting in the recruitment and activation of ZAP-70 (26, 28). ZAP-70 activation leads to the recruitment and phosphorylation of several adaptor proteins, such as the linker for activation of T cells (LAT; ref. 29), thereby coupling the TCR to downstream activation signals. Inhibition of T-cell activation by anti-CD4 has thus been suggested to occur by inhibiting the necessary p56^{lck}-mediated costimulation (30).

In the present study, we describe the inhibition of T cells by zanolimumab both *in vivo* and *in vitro* and show that this therapeutic mAb exerts its inhibitory actions via several different, nonexclusive, and sequential mechanisms.

Materials and Methods

Antibodies and Glutathione S-Transferase Construct

Zanolimumab [HM6G.2, HuMax-CD4; fully human IgG1 (hIgG1), κ antibody; ref. 11] was manufactured by Medarex and DSM Biologics; F(ab')₂

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and Fab fragments were prepared by Genmab B.V. Control hIgG1 was from Sigma. HuMab-KLH was produced by Genmab B.V. OKT3 was purified from hybridoma supernatant (all experiments except CDC), and mouse anti-human CD3, clone OKT3, was from Ortho Biotech (CDC). CD28.2 was from BD PharMingen. Anti-CD45RA-APC, anti-CD45RO-PE, and anti-human CD4 mAb (MT-477-FITC or PE) were from BD PharMingen; rabbit anti-human C1q-FITC and rabbit anti-human C4c-FITC were from DAKO. AKT, phospho-AKT (S-473), extracellular signal-regulated kinase 1/2 (Erk1/2), phospho-Erk1/2 (T-202/Y204), glutathione S-transferase (GST), p38, phospho-p38 (T-180/Y-182), phospho-Src (Y-416), Lck, phospho-SHIP (Y-1021), ZAP-70, and phospho-ZAP-70 (Y-319) were from Cell Signaling. LAT, phosphotyrosine (4G10), and SHIP mAb were from Upstate Biotechnology. TCR ζ chain was from Zymed Laboratories. Dok-1 rabbit anti-serum was prepared in-house (J.C.). Rabbit CD4 anti-serum was kindly provided by Mark Marsh (Imperial College, London, United Kingdom). Horseradish peroxidase-conjugated secondary antibodies were from DAKO. The SH2C-RasGAP-GST construct is described by Duchesne et al. (31) and was kindly supplied by J. Nunes (Institut Paoli-Calmettes, Marseille, France).

Clinical Study Designs

Psoriasis. A multicenter, double blind, randomized, placebo-controlled, parallel-group phase II study of zanolimumab was conducted in patients with moderate to severe psoriasis vulgaris. The patients were randomized to either placebo, 40 mg zanolimumab, 80 mg zanolimumab, or 120 mg zanolimumab s.c. once weekly for 14 weeks.

Cutaneous T-cell lymphoma. The efficacy and safety of zanolimumab in patients with refractory CTCL have been assessed in two, phase II, multicenter, prospective, open-label, uncontrolled clinical studies. Patients with treatment refractory CD4⁺ CTCL (mycosis fungoides, $n = 38$; Sézary syndrome, $n = 9$) received 17 weekly infusions of zanolimumab (early-stage patients, 280 or 560 mg; advanced-stage patients, 280 or 980 mg). Included are the data available from this study.

Leukocytes characterized by the following immunologic markers were assessed by means of flow cytometry (FACScan, Becton Dickinson): CD3⁺ T cells, CD3⁺CD4⁺ T cells. Staining for CD4 was done with RPA-T4, a mAb directed against a distinct CD4 epitope and noncompetitive with zanolimumab.

Complement Deposition on Cells

CD4⁺ T cells were purified via negative selection using magnetic beads (Dyna T Cell Negative Isolation kit, Dynal Biotech GmbH) from peripheral blood mononuclear cells (PBMC) isolated by lymphoprep density centrifugation from human peripheral blood or buffy coats. Next, cells were incubated with various concentrations of zanolimumab or OKT3 mAb. For analysis of C1q binding or C4c deposition, cells were incubated with human serum followed by staining with FITC-labeled C1q or C4c mAb. For determination of preferential binding of complement to CD45RA⁺ and CD45RO⁺ cells, cells were also stained with the appropriate (fluorochrome labeled) CD45 mAb. Cell-associated fluorescence was detected by flow cytometry (FACSCalibur, Becton Dickinson).

Antibody-Dependent Cell-Mediated Cytotoxicity

Total CD4⁺ T cells, CD45RO⁺ and CD45RA⁺ subsets, and natural killer (NK) cells were isolated from blood bank leukopheresis packs obtained from healthy donors of both sexes (National Blood Service, Brentwood, Essex, United Kingdom). CD4⁺ T cells or NK cells were isolated using the appropriate enrichment RosetteSep cocktail (25 μ L/mL; Stemcell Technologies, Inc.). Cells were separated by lymphoprep (Axis Shield) and resuspended in RPMI 1640 containing 1% penicillin and streptomycin and rested for 1 h at 37°C, 5% CO₂. CD45RA⁺ and CD45RO⁺ T-cell subsets were isolated by negative selection using the relevant StemSep human CD4⁺ T-cell enrichment kit (Stemcell Technologies). Cell purity was routinely >90%.

ADCC was done using autologous purified T cells and NK cells using zanolimumab or a control hIgG1 as described previously (32).

CD4 Receptor Down-modulation

The capacity of zanolimumab to down-regulate CD4 in the presence of effector cells was studied with PBMC-derived CD4⁺ T cells (for isolation, see

CDC) or SUP-T1 cells and with or without the addition of PBMC-derived monocytes (Dyna Monocyte Negative Isolation kit) or TPH-1 cells. PBMC or SUP-T1 cells were incubated with a concentration range of zanolimumab, zanolimumab-F(ab)₂ fragments (SUP-T1), zanolimumab-Fab (SUP-T1), or the negative control HuMab-KLH. When appropriate, effector cells were added to an effector cell:target cell ratio of 10:1, 5:1, or 4:1. IFN γ (concentration range, 125–1,000 μ g/mL) was added and cells were incubated overnight. Cells were stained for CD4 with fluorochrome-labeled M-T477 (noncompetitive antibody from BD PharMingen) and a target cell selection marker. Cell-associated fluorescence was assessed by flow cytometry.

Proliferation Assays

Tetanus toxin-specific T-cell proliferation. PBMC (for isolation, see CDC) were stimulated with 10 μ g/mL tetanus toxoid, incubated with or without zanolimumab for 5 or 6 days, and incubated with 0.5 μ Ci/well [³H]thymidine (Amersham Biosciences) for 18 to 20 h. Cells were harvested (Filter Mate Cell harvester, Packard, via Perkin-Elmer) and [³H]thymidine incorporation was measured (Topcount NXT microplate scintillation counter, Packard).

OKT3 and OKT3/anti-CD28 induced T-cell proliferation. Tissue culture plates were coated with OKT3 with or without 1 μ g/mL anti-CD28 overnight at 4°C and washed once with PBS, and 2×10^5 per well CD4⁺ T cells were added (for isolation method, see ADCC). Soluble zanolimumab was added, and cells were incubated at 37°C for 48 h, then pulsed with 1 μ Ci [³H]thymidine for a further 24 h, and harvested onto glass fiber filter plates (Perkin-Elmer Instruments). [³H]thymidine incorporation was measured by scintillation counting. Samples for cytokine assays were removed at 48 h.

Activation Marker Expression

CD4⁺ T cells (10^6) were incubated on 0.1 or 0.5 μ g/mL plastic immobilized OKT3 for 72 h with or without zanolimumab. Cells were resuspended and washed twice in fluorescence-activated cell sorting buffer, stained with CD25 FITC and CD69PE mAb (BD Biosciences), and analyzed by flow cytometry.

Cell Stimulation and Immunoprecipitation

CD4⁺ T cells (1×10^7 for whole-cell lysates or 3×10^7 for immunoprecipitates) were preincubated with 20 μ g/mL zanolimumab for 10 min at 37°C. Cells were washed once before stimulation with 10 μ g/mL anti-CD3 mAb (OKT3) or isotype control immobilized onto 0.1- μ m latex beads (Sigma) at 37°C for the indicated time points. Samples were pelleted and lysed as described by Harding et al. (30). Lysates were precleared using protein G-Sepharose (Amersham Biosciences) and incubated with immunoprecipitating antibody overnight at 4°C before precipitation with protein G-Sepharose. For SH2C-RasGAP-GST precipitations, lysates were precleared using glutathione agarose beads (Amersham) bound to an empty GST vector. Cleared lysates were added to 10 μ L glutathione agarose beads bound to 10 μ g SH2C-RasGAP-GST and incubated overnight with inversion at 4°C. Beads were washed four times in lysis buffer before SDS-PAGE analysis. For experiments using the Src kinase inhibitors PP2 or damnacanthal (Merck Biosciences), cells were preincubated with the inhibitor for 45 min at 37°C before exposure to zanolimumab for 5 min before lysis and Dok-1 precipitation.

Immunoblotting

Immunoprecipitates were separated by 7% to 15% gradient gel SDS-PAGE and immunoblotting was done as described by Harding et al. (30).

In vitro Kinase Assays

CD4-associated p56^{lck} *in vitro* tyrosine kinase assays were carried out by incubating CD4⁺ T cells with zanolimumab for the indicated time period before lysis and removal of debris as described above. CD4 was immunoprecipitated using protein G-Sepharose, and kinase assays were done as described by Harding et al. (30). Dried membranes were exposed for autoradiography. Once decayed, the membranes were probed with the indicated antibodies to measure loading.

Results

Depletion of CD3⁺CD4⁺ cells *in vivo*. Zanolimumab induces a strong depletion of CD4⁺ T cells as shown in clinical trials in patients suffering from psoriasis and CTCL (Fig. 1). Interestingly, effective and sustained depletion from peripheral blood required repeat dosing. In psoriasis patients exposed to zanolimumab by weekly s.c. dosing with 40, 80, or 120 mg antibody for 14 weeks (Fig. 1A), a gradual decline in CD4 T-cell counts was observed with maximum depletion occurring after about 6 weeks after start of the treatment. In CTCL, patients were exposed to higher doses of i.v. zanolimumab (early-stage patients, 280 or 560 mg; advanced-stage patients, 280 or 980 mg). In this study, depletion kinetics seemed faster with maximum depletion occurring 2 to 3 weeks following initiation of the treatment (Fig. 1B).

Fc-dependent effector mechanisms: CDC, ADCC, and CD4 down-modulation. To analyze the mechanisms of action of zanolimumab, we did studies using primary T cells. The use of primary T cells is important as previous studies have shown important differences in the activity of CD4 mAb against primary T cells and immortalized T-cell lines (19). First, CDC by zanolimumab was assessed. This, however, did not mediate cell death of CD4⁺ T cells nor CD45RA⁺ or CD45RO⁺ T-cell subsets investigated separately (data not shown). The lack of CDC could be explained by a poor ability to induce classic pathway activation. Although the antibody, being a hIgG1, retained the intrinsic ability to activate complement as measured in a plate-bound assay, it neither bound C1q (data not shown) nor fixed complement factors C4 (Fig. 2A) or C3 (data not shown) when bound to the surface of CD4⁺CD45RA⁺ or CD4⁺CD45RO⁺ primary T cells. In contrast, a control mAb against CD3 (OKT3) showed effective activation of complement via the classic pathway, shown by the deposition of C4 on the T-cell surface (Fig. 2A).

Second, we investigated the ability of zanolimumab to induce ADCC using NK cells as effector cells. We found that zanolimumab is very effective in promoting killing of CD4⁺ T cells with significant specific lysis already occurring at 10 ng/mL (Fig. 2B). Zanolimumab promoted a stronger killing of the CD45RO⁺ subset than the CD45RA⁺ subset at concentrations of 10 and 100 ng/mL (Fig. 2B).

Third, we examined the ability of zanolimumab to induce CD4 down-regulation. CD4 expression was investigated by flow cytometry with a noncompeting CD4 mAb using freshly isolated CD4⁺ T cells or SUP-T1 T cells. Zanolimumab dose-dependently down-regulated CD4 from purified primary CD4⁺ T cells (Fig. 3A) or SUP-T1 T cells (Fig. 3B), requiring the presence of IFN γ -activated monocytes or a monocytic cell line. The results showed that after 18 to 24 h, CD4 expression was reduced by 50% to 80%. Down-modulation seemed to be Fc dependent, as incubation with zanolimumab F(ab')₂ fragments did not result in a reduction in CD4 expression (Fig. 3B). At high mAb concentrations, soluble zanolimumab failed to down-regulate CD4, possible due to monomeric binding, resulting in a reduction of cross-linking. Cross-linking via immobilized zanolimumab or zanolimumab captured on a plate-bound anti-IgG antibody also down-regulated CD4 (data not shown).

CD3-induced T-cell activation is inhibited by direct actions of CD4 mAb. Proliferation assays were carried out using purified primary CD4⁺ T cells and direct inhibitory effects of zanolimumab were studied on cells responding to tetanus toxin and immobilized anti-CD3 mAb OKT3. Zanolimumab was a potent inhibitor of TCR-stimulated T-cell proliferation both in antigen-dependent (tetanus toxin induced; Fig. 4A) and in antigen-independent (anti-CD3 induced) T-cell proliferation (Fig. 4B). Similar inhibitory effects on anti-CD3-induced T-cell proliferation were found for interleukin (IL)-2 and IL-4 production (Fig. 4C). Optimal inhibition of production of these cytokines occurred at saturating concentrations of 1 μ g/mL zanolimumab. It has been shown that CD28 costimulation may prevent the inhibition of T-cell activation by CD4 antibodies (33, 34). We therefore carried out proliferation assays with OKT3 in the presence of immobilized CD28 mAb. Figure 4B shows that proliferation was still inhibited by zanolimumab, albeit to a somewhat lesser extent than without CD28 costimulation. Thus, CD28 costimulation was unable to effectively prevent the inhibitory actions of zanolimumab.

Finally, experiments were carried out to assess the inhibition of T-cell activation by examining expression of activation markers. This study shows a reduction in the fraction of CD4⁺ T cells expressing CD25 and CD69 after 72 h of OKT3 stimulation in the

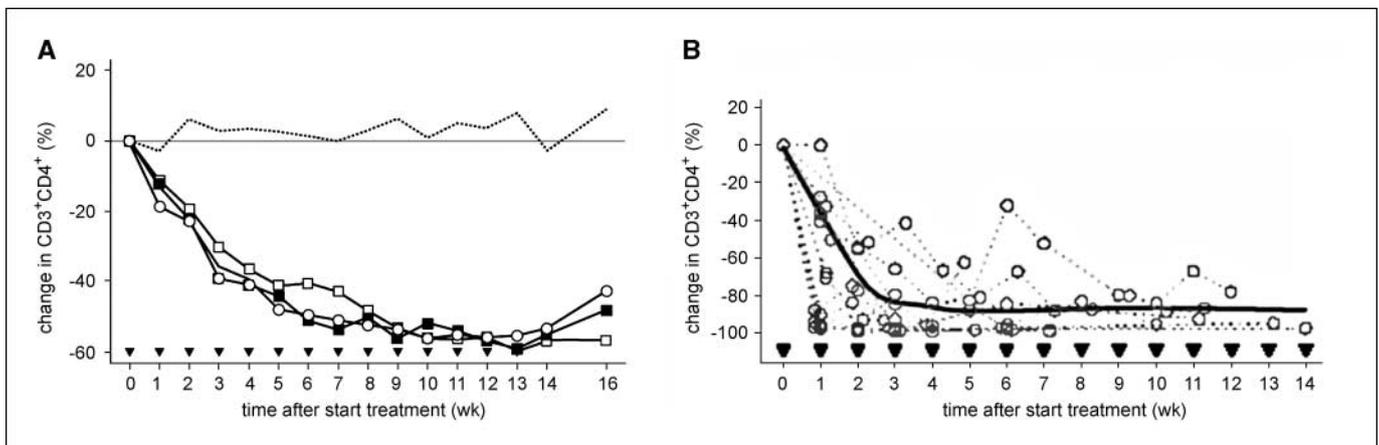


Figure 1. Treatment of patients with zanolimumab leads to CD3⁺CD4⁺ T-cell depletion in peripheral blood. A, weekly s.c. treatment of psoriasis patients with zanolimumab (14 wk). Data are the mean percentage decrease (compared with the baseline) of peripheral blood CD3⁺CD4⁺ T cells for patients treated with 40 mg (□), 80 mg (■), or 120 mg (○). B, weekly i.v. treatment of CTCL patients with zanolimumab (first 14 wk of treatment shown). Data are the mean percentage decrease (compared with the baseline) of peripheral blood CD3⁺CD4⁺ T cells (solid line). Open circles connected by dotted lines, the percentage decrease (compared with the baseline) of peripheral blood CD3⁺CD4⁺ T cells for individual patients.

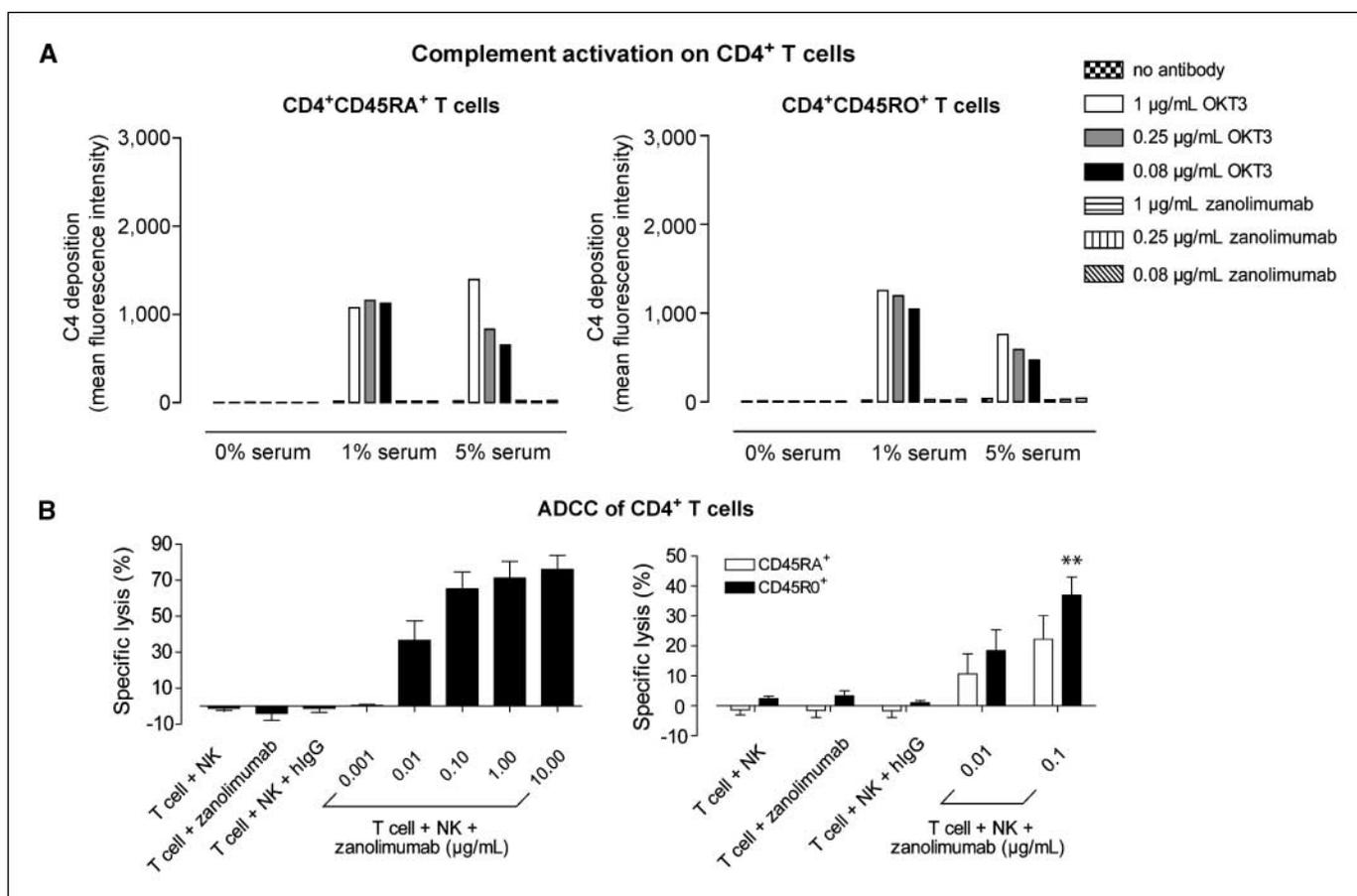


Figure 2. Zanolimumab selectively kills CD4⁺ T cells by ADCC. *A*, zanolimumab does not fix complement factor C4 when bound to CD4⁺ T cells. Zanolimumab bound to cells does not induce complement activation as measured by deposition of C4. Representative of three independent experiments. The CD4⁺CD45RA⁺ T-cell population (*left*) and the CD4⁺CD45RO⁺ T-cell population (*right*) were measured using anti-CD45RA⁺-PE and CD45RO⁺-APC antibodies. The antibody OKT3, as positive control at the same concentrations, does induce C4 deposition. *B*, zanolimumab kills CD4⁺ T cells via ADCC. CD4⁺ T cells are efficiently killed by NK cell-dependent ADCC (*left*). *Left*, histograms of the CD4⁺ T cells. *Columns*, mean of four independent experiments; *bars*, SE. *Right*, histograms of the CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺ T cells. *Columns*, mean of nine independent experiments; *bars*, SE. **, $P < 0.005$, Wilcoxon signed-rank test, CD4⁺CD45RO⁺ specific lysis by zanolimumab differs significantly from CD45⁺CD45RA⁺ specific lysis.

presence of zanolimumab. The percentage of cells expressing CD69 or CD25 was inhibited by up to approximately 40% to 50% (data not shown).

Rapid inhibition of TCR signal transduction by zanolimumab. TCR stimulation results in the induction of tyrosine phosphorylation of a wide range of intracellular proteins. Figure 5*A* (*top*) shows that exposure of primary T cells to soluble zanolimumab followed by TCR stimulation (immobilized CD3 mAb) caused a generalized inhibition of protein phosphorylation within minutes. Analysis of whole-cell lysates in immunoblots, probed for phosphotyrosine, showed that phosphorylation of proteins of 21 and 23 kDa was markedly inhibited. Immunoblotting with an antibody specific for TCR ζ identified these proteins as the p21 and p23 phosphoisomers of TCR ζ , with the proportion of phosphorylated TCR ζ being small compared with the nonphosphorylated fraction of this protein (migrating at 16 kDa; Fig. 5*A*, *bottom*). The TCR-stimulated generation of both phosphoisomers was reduced by ~50% by exposure to zanolimumab (calculated by assessing densitometric values normalized for loading, relative to values observed after 2 min of CD3 stimulation in the absence of zanolimumab). Because phosphorylation of the TCR ζ chain is critical for activation of the downstream tyrosine kinase ZAP-70 (26), we assessed ZAP-70 activation by measuring phosphorylation

of Tyr³¹⁹. Figure 5*B* shows that zanolimumab caused a reduction of 50% in TCR-stimulated ZAP-70 phosphorylation at Tyr³¹⁹, comparable with the extent of inhibition of TCR ζ phosphorylation.

Next, we investigated three downstream signaling pathways that play critical roles in T-cell activation and their differentiation into effector populations. Figure 5*C* shows that zanolimumab inhibited activation of both the Erk1/2 and p38 serine/threonine kinase pathways to a comparable extent (~50%), although inhibition of the Erk1/2 pathway was only reproducibly apparent at later time points (5–30 min). The AKT/protein kinase B kinase signaling pathway, downstream of phosphatidylinositol 3-kinase, was also investigated. Figure 5*C* shows that CD3 stimulation of AKT, assessed by immunoblotting of its activation-dependent phospho-Ser⁴⁷³ phosphorylation site, was inhibited by ~50% following T-cell exposure to zanolimumab. In summary, zanolimumab inhibits the very earliest CD3-stimulated activation events by ~50%, and a comparable level of inhibitory action is thereby transmitted to multiple downstream signaling pathways, including Erk1/2, p38, and AKT.

Zanolimumab stimulates CD4-associated p56^{lck}, Dok-1, and SHIP-1 phosphorylation. Given that CD4 ligation has long been known to activate the p56^{lck} tyrosine kinase (35), it might seem counterintuitive that zanolimumab causes a marked reduction in

two well-established $p56^{\text{lck}}$ substrates: TCR ζ and ZAP-70 (Fig. 5). We therefore examined $p56^{\text{lck}}$ kinase activation kinetics following binding of zanolimumab and compared them to activation through anti-CD3 stimulation. Interestingly, zanolimumab caused rapid stimulation (2–2.5 times maximal increase) of CD4-associated $p56^{\text{lck}}$ tyrosine kinase activity following CD4 ligation, including $p56^{\text{lck}}$ autophosphorylation (Fig. 6A, *top*). The rapid activation of phosphorylation by anti-CD4 binding was confirmed by assessing phosphorylation of α -casein as an exogenous substrate (Fig. 6A, *middle top*). Optimal stimulation was apparent at 5 min, comparable with the time at which optimal CD3-induced TCR ζ phosphorylation was observed (cf. Fig. 5A). Consistent with these results, probing of the membrane using a phosphospecific antibody showed that phosphorylation of the phospho-Tyr³⁹⁴ autophosphorylation site of $p56^{\text{lck}}$ was optimal (3.7 times increase) at 5 min (Fig. 6A, *middle*). Reprobing for Lck and CD4 enabled values to be quantified and normalized for loading (Fig. 6A, *middle bottom and bottom*). In conclusion, CD4 ligation using zanolimumab causes $p56^{\text{lck}}$ activation with kinetics very similar to those observed when a CD3 mAb is used to stimulate $p56^{\text{lck}}$ -dependent intracellular protein tyrosine phosphorylation (Fig. 5).

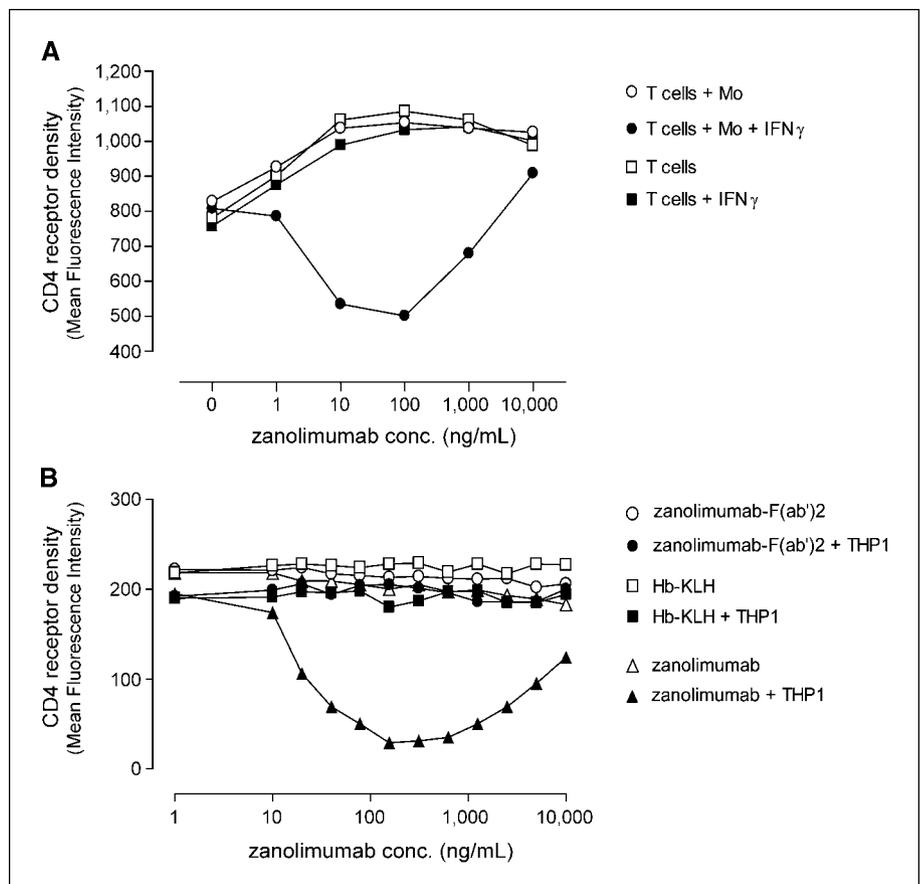
As zanolimumab increased $p56^{\text{lck}}$ activity 2- to 3-fold, whereas phosphorylation of $p56^{\text{lck}}$ substrates TCR ζ and ZAP-70 was decreased (Fig. 5A and B), we hypothesized that the activated kinase couples directly to one or more inhibitory signaling pathways. Potential candidates for such regulation are the adaptor molecule Dok-1, thought to be involved in down-regulating Ras-mitogen-activated protein kinase pathways (36) and the inositol 5-phosphatase SHP-1. Phosphorylated Dok-1 was precipitated

using a GST construct containing the COOH-terminal SH2 domain of RasGAP, which only binds to Dok-1 when it is phosphorylated. Figure 6B shows that zanolimumab induced significant Dok-1 tyrosine phosphorylation (~6-fold increase; calculated by densitometric values normalized for GST loading) within 5 min of CD4 ligation. Furthermore, SHP-1 phosphorylation was also enhanced up to 3-fold (densitometric values normalized for SHP-1 loading; Fig. 6C). Previous findings suggest that Dok-1 association with RasGAP is dependent on $p56^{\text{lck}}$ tyrosine kinase activity (37). We therefore incubated cells with the Src inhibitor PP2 and the more specific $p56^{\text{lck}}$ inhibitor damnacanthal and found that the amount of Dok-1 that precipitated with SH2C-RasGAP-GST was reduced by 60% or more (Fig. 6D). Overall, these results indicate that CD4 ligation with zanolimumab causes $p56^{\text{lck}}$ kinase activation, resulting in increased Dok-1 and SHP-1 phosphorylation, thereby coupling CD4 to pathways that lead to direct inhibitory effects of CD4 mAbs on T-cell activation.

Discussion

The fully human CD4 antibody, zanolimumab, has been assessed in clinical trials in inflammatory diseases, including rheumatoid arthritis and psoriasis, in which it induced a profound T-cell depletion. Zanolimumab was found to be safe and well tolerated. The effective *in vivo* depletion of CD4⁺ cells in humans makes zanolimumab a relevant candidate for the development of immunotherapeutic approaches against T-cell lymphomas. Zanolimumab has already shown promising results in immunotherapy of CTCL with a response rate at the higher doses of 50% to 75% and

Figure 3. Zanolimumab down-modulates CD4 expression. **A**, dose-response plots of CD4 expression were carried out using purified blood CD4⁺ T cells incubated for 18 h with zanolimumab in the presence or absence of monocytes (*Mo*) incubated with or without IFN γ . **B**, dose-response plots of CD4 expression were carried out using CD4⁺ CEM-NKr cells incubated for 18 h with zanolimumab, F(ab)₂ fragments of zanolimumab, or an isotype control antibody (HuMab-KLH) in the presence or absence of THP-1 cells. The CD4 expression levels were measured using a noncompeting CD4 antibody, MT-477. Results are representative of four different experiments.



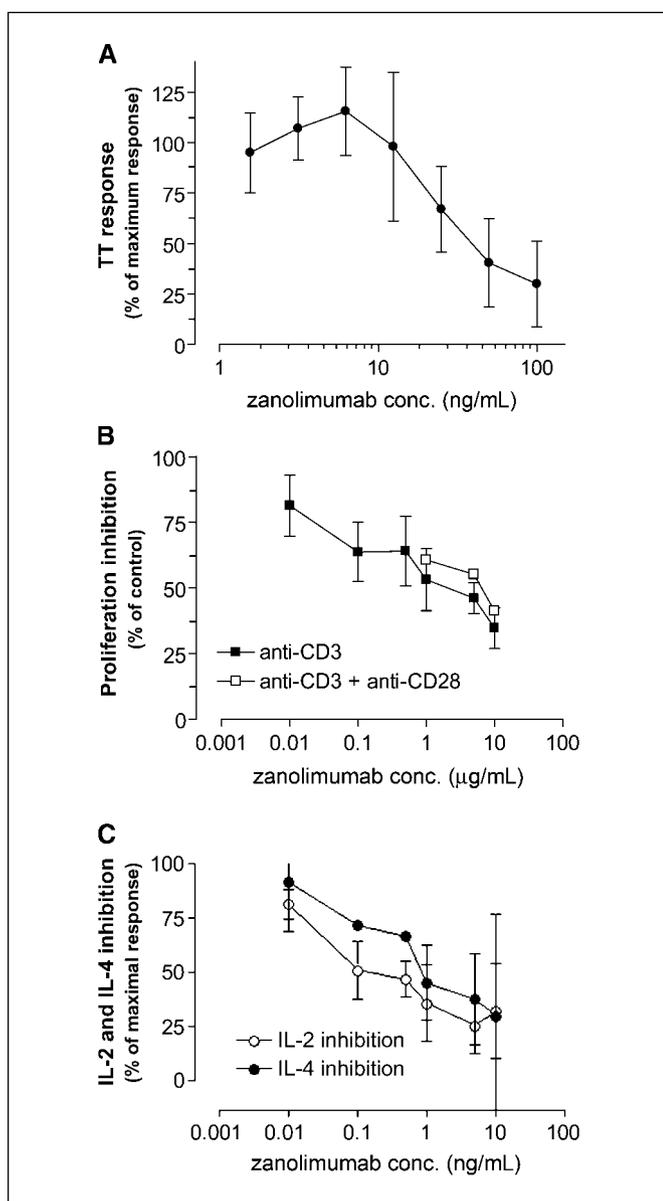


Figure 4. Zanolimumab inhibits T-cell activation. *A*, dose-response plots of proliferation assays were carried out using purified CD4⁺ T cells stimulated with tetanus toxoid (TT; 10 µg/mL) at the concentrations shown. Points, mean of two independent experiments; bars, SE. *B*, dose-response plots of proliferation assays were carried out using purified CD4⁺ T cells stimulated with immobilized OKT3 mAb (0.1 µg/mL) coated onto latex beads, either with or without the addition of anti-CD28 (1.0 µg/mL) at the concentrations shown. Points, mean of four independent experiments; bars, SE. *C*, supernatants removed from proliferation assays after 48 h were assayed for IL-2 and IL-4. Points, mean of four independent experiments; bars, SE.

median response duration of more than 10 months (13) and is currently in clinical development in CTCL (pivotal study) and nodal T-cell lymphoma (phase II). The present study shows that zanolimumab kills and inhibits T cells via several different but nonexclusive mechanisms. Zanolimumab might intervene in the pathogenesis of CTCL both by depleting CD4⁺ tumor cells and by inhibiting the interaction of dendritic cells and lymphoma cells residing in the skin, an interaction suggested to be critical for continued tumor cell activation (38). Direct inhibitory effects on T-cell signaling pathways, as well as induction of Fc-mediated

effector function, may therefore play a role in the clinical efficacy of this therapeutic mAb.

A difference is noted in the rate of CD4⁺CD3⁺ T-cell depletion between psoriasis patients treated with 40 to 120 mg zanolimumab (s.c.) once weekly and CTCL patients treated with 280 to 960 mg zanolimumab (i.v.) once weekly. Although it cannot be excluded that differences between patient groups or route of administration affect the depletion observed, it is most likely that the higher dosing in CTCL patients is mainly responsible for the more rapid depletion. Indeed, a subanalysis of CTCL patients dosed with 280 mg zanolimumab compared with patients dosed with 560 or 960 mg showed a stronger decline in peripheral blood T cells at the higher doses (13).

The inhibitory mechanisms of action of zanolimumab include both very rapid mechanisms leading to immediate inhibition of signaling, observed within minutes following binding, and Fc-dependent mechanisms, acting with intermediate (hours) to slow kinetics (days). Zanolimumab does not bind or activate complement when bound to CD4 on the surface of primary T cells, although complement-binding ELISAs indicate that the Fc part of zanolimumab has an intrinsic capability to interact with complement. Interestingly, poor recruitment of complement has been described for all known CD4 mAbs of the hIgG1 isotype, including the chimerized, primatized, or humanized CD4-specific antibodies: priliximab, keliximab, and mAb 4162W94 (16–18). Several of these CD4 antibodies recognize epitopes similar to leu3a, a murine antibody that induces CDC efficiently (15). This difference between human and murine CD4 mAb is currently not understood. However, it is well known that differences in antigen fine specificity may result in dramatic differences in the ability of mAb to induce complement-mediated lysis as, for example, shown for CD20 mAb (39).

In contrast, zanolimumab induces potent ADCC of both CD45RA⁺ and CD45RO⁺ subpopulations of primary CD4⁺ T cells at relatively low concentrations. We noted that CD45RO⁺ primary T cells were more sensitive to NK-mediated killing *in vitro* than naive CD45RA⁺ cells (Fig. 2). This increased sensitivity correlates with a more profound depletion of CD45RO⁺ T cells *in vivo* as reported in patients who were exposed short-term (4 weekly infusions) to zanolimumab (12). Following longer-term treatment (14 weeks), maximal depletion of both subsets was similar. Consistent with the short-term treatment results, however, depletion of CD45RO⁺ cells occurred more rapidly compared with CD45RA⁺ cells (data not shown). Differences between T-cell subsets in susceptibility to ADCC have been observed before. Previously, we have shown that CD4⁺CD45RO⁺ T cells are also more sensitive to ADCC by alefacept, a fusion protein of the extracellular domain of leukocyte function antigen-3 and the Fc portion of IgG1 (32). One possible explanation for our findings is that CD45RO⁺ T cells express more ligand for a NK cell-activating receptor and/or less ligand for a NK cell inhibitory receptor than CD45RA⁺ T cells. Further work is required to elucidate the underlying mechanism, which may be relevant for differences in response rates to zanolimumab between patients.

The zanolimumab-induced partial CD4 down-regulation seems to require CD4 clustering on the T-cell surface, which can be artificially induced by plate-immobilized zanolimumab at high concentrations or by zanolimumab in the presence of FcR-bearing effector cells. Most IgG1 isotype CD4 mAbs down-modulate CD4 over time (16–19). It has been suggested that receptor modulation can be caused by internalization (40), as well as by stripping of CD4 from cell surfaces (16). The ability to down-modulate CD4

expression varies among CD4 antibodies. The Fc-dependent downmodulation of CD4 from the cell surface observed in the presence of zanolimumab could provide long-term inhibition of T-cell signaling and thereby benefit antitumor therapy, as described, for example, for epidermal growth factor receptor mAb (41). In Fig. 3, it is noted that CD4 expression on primary T cells, but not on SUP-T1 cells, seems to increase on incubation with zanolimumab in the absence of cross-linking. A difference between SUP-T1 compared with primary T cells is its very low expression of CD3/TCR. We have shown above that zanolimumab sequesters CD4 away from the CD3/TCR complex. It may be that this sequestration on primary T cells increases the availability of the epitopes for the CD4-detecting antibody MT-477.

Zanolimumab inhibits T-cell activation by two distinct pathways. First, it exerts a negative inhibitory effect on one of the earliest biochemical events in the TCR signaling cascade, the phosphorylation of ITAM signaling motifs within the TCR ζ chains (Fig. 5A), consistent with our previous findings using YHB.46 (34). It seems that once TCR ζ phosphorylation is reduced by ~50%, multiple downstream signals are likewise inhibited to a comparable extent

(Figs. 4C and 5C). Intriguingly, independent CD4 ligation induces an enhanced stimulation of CD4-associated p56^{lck} kinase activity over the exact time span at which CD3-stimulated TCR ζ and ZAP-70 phosphorylation is most inhibited, suggesting that the activated p56^{lck} does not have effective access to its TCR ζ and ZAP-70 substrates. These data are consistent with sequestration of CD4 and a pool of associated p56^{lck} molecules, which can then no longer be recruited into juxtaposition with the TCR.

Is the ~50% inhibition of TCR ζ phosphorylation sufficient to explain all the inhibitory effects exerted on T-cell activation by zanolimumab? This seems unlikely. It is therefore of importance that our findings also provide support for inhibition of T cells via the transmission of direct inhibitory intracellular signals following CD4 binding. Treatment of T cells with zanolimumab induced phosphorylation of both Dok-1 and SHIP-1 (Fig. 6B and C). It has been reported that Dok-1 and p56^{lck} are associated via an SH2-mediated interaction, which results in phosphorylation of the Dok-1 molecule (42). In the present work, Dok-1 phosphorylation was reduced by p56^{lck} kinase inhibitors (Fig. 6D), consistent with the notion that CD4-stimulated p56^{lck} causes the increased Dok-1 phosphorylation.

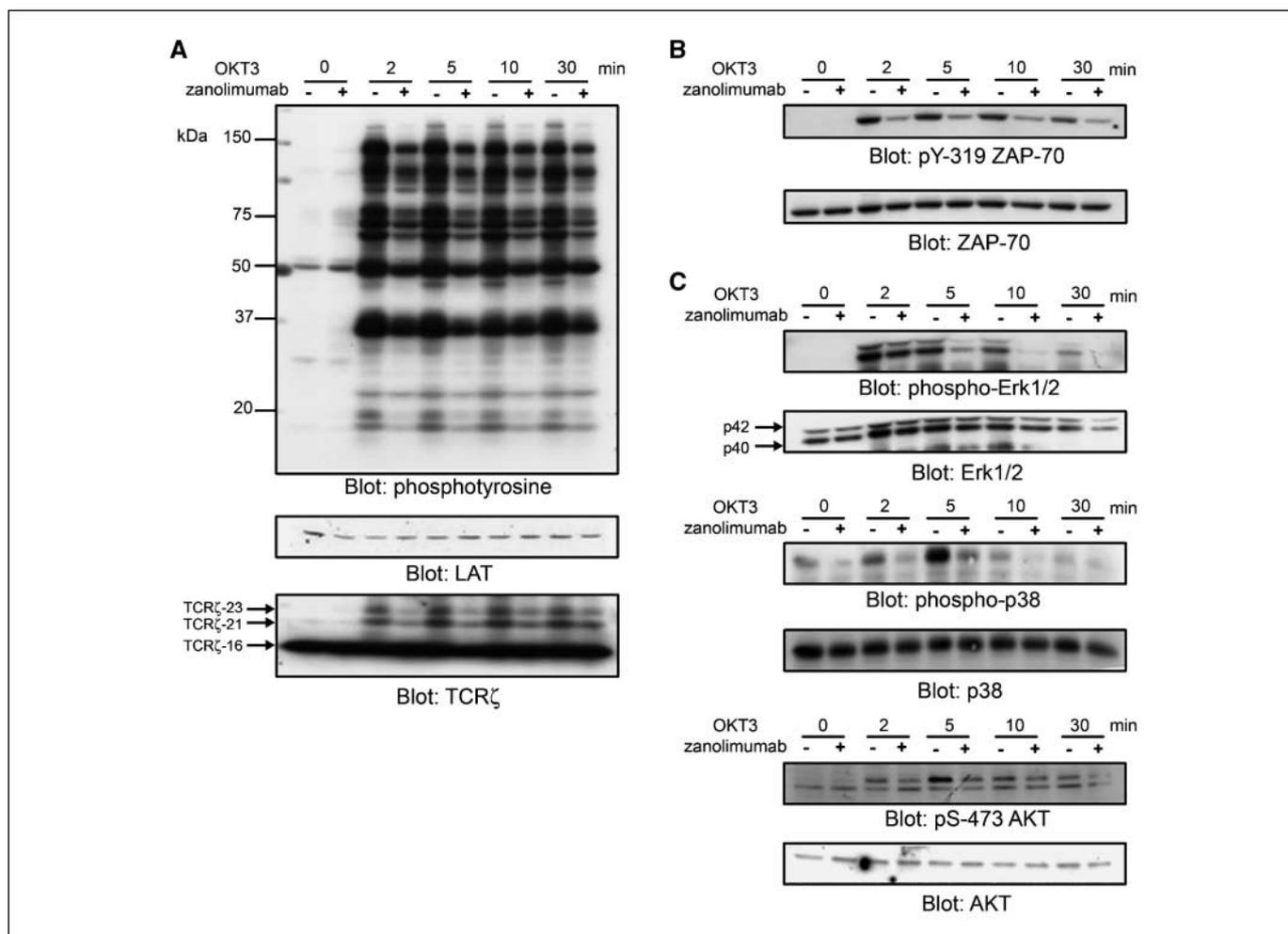


Figure 5. Zanolimumab inhibits initiation of TCR signal transduction and downstream signals in CD4⁺ T cells. Purified CD4⁺ T cells were incubated with (+) or without (-) zanolimumab for 10 min before incubation with mouse IgG1 (0 min) or OKT3-coated latex beads at 37°C for the times shown. Cells were lysed before samples were split into two and proteins were separated by 7% to 15% gradient SDS-PAGE. A, one membrane was then immunoblotted with the phosphotyrosine mAb 4G10 before stripping (top) and reprobing with LAT (middle) and TCR ζ antibodies (bottom). B and C, the second membrane was probed with phospho-ZAP-70 (B) and phospho-Erk1/2 antibodies, phospho-p38, and phospho-AKT (C) and followed by stripping and reprobing with ZAP-70, Erk1/2, p38, and AKT, respectively, to assess loading. Results for the experiments are representative of four different experiments.

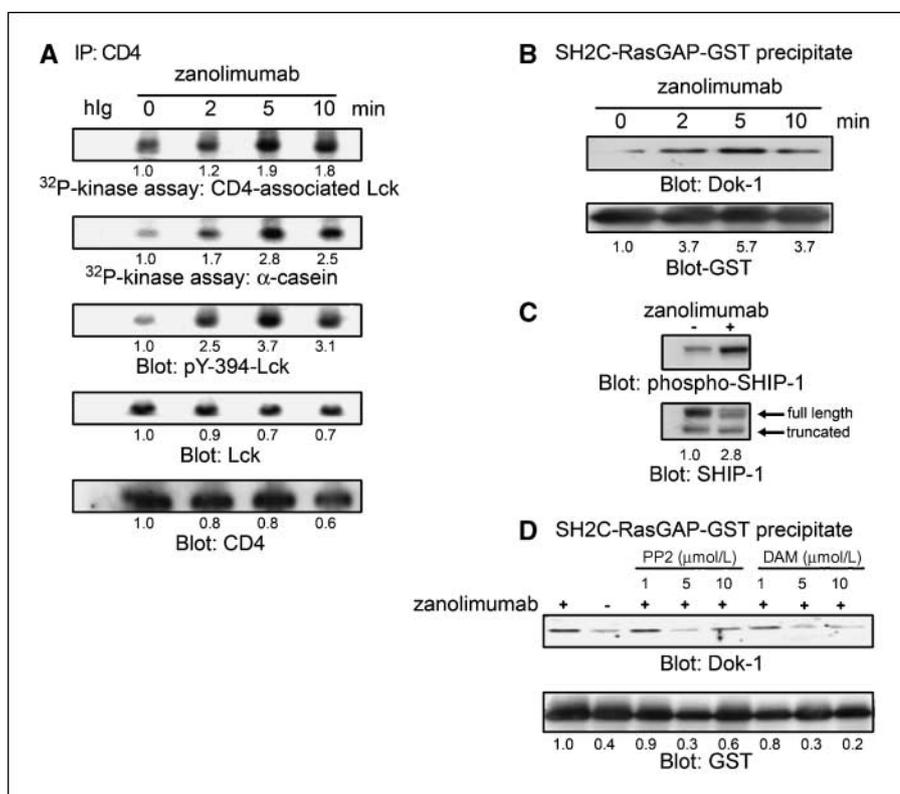


Figure 6. Zanolimumab induces p56^{lck}-dependent phosphorylation of Dok-1 and SHIP-1. **A**, CD4-associated p56^{lck} tyrosine kinase activity is increased after CD4 ligation. Purified CD4⁺ T cells were incubated with zanolimumab, and CD4 was then immunoprecipitated from cell lysates, followed by *in vitro* kinase assays in the presence of α -casein before transfer to an immunoblotting membrane. CD4-p56^{lck} autophosphorylation (*top*) and phosphorylated α -casein (*middle top*). Membranes were blotted for pY-394-p56^{lck} and stripped and reprobed for both p56^{lck} and CD4 as loading controls (*middle, middle bottom, and bottom*). **B**, Dok-1 phosphorylation is increased after CD4 ligation. Cells treated with zanolimumab were lysed and precleared with glutathione agarose beads coupled to an empty GST construct before Dok-1 was precipitated using a GST construct of the COOH-terminal SH2 domain of RasGAP (SH2C-RasGAP-GST). Samples were blotted with Dok-1 and GST antibody. **C**, SHIP-1 phosphorylation is increased after CD4 ligation. Cells were treated with either 10 μ g/mL zanolimumab for 10 min before immunoblotting whole-cell lysates with a phosphospecific SHIP-1 antibody. **D**, phosphorylation of Dok-1 is dependent on the action of Src family kinases. CD4⁺ T cells were preincubated with the Src family inhibitors PP2 or damnacanthal (DAM) for 45 min before treatment with zanolimumab for 5 min and Dok-1 phosphorylation was then measured as in **C**. Results are representative of four different experiments.

Activation of Dok-1 and SHIP-1 may inhibit CD4⁺ T-cell activation processes in two possible ways. First, SHIP-1 is known to dephosphorylate PI(3,4,5)P₃, generating PI(3, 4)P₂ (43). Because AKT requires PI(3,4,5)P₃ for its targeting to the plasma membrane and full activation (44), SHIP-1 stimulation should inhibit the AKT pathway, consistent with our findings (Fig. 5C). This is comparable with the Fc γ RIIB signaling pathway in which SHIP-1 has likewise been shown to inhibit AKT activation (45). The significance of inhibition of AKT stimulation for activation is that this pathway has been implicated in the induction of transcription factors critical for *IL-2* gene induction, such as NFAT, activator protein-1, and nuclear factor- κ B (46–48). Second, the activation of SHIP-1 and Dok-1 could lead to dysregulation of cytoskeletal changes (22, 32, 35, 45, 49), perhaps explaining the inability of CD4-p56^{lck} to target its normal kinase substrates following CD4 ligation. Taken together, our findings on Dok-1 and SHIP-1 stimulation provide attractive mechanisms that explain the rapid and potent inhibitory actions of zanolimumab following CD4 ligation and consequent p56^{lck} activation.

Overall, the present study suggests that zanolimumab uses several distinct and sequential mechanisms of action, all of which have the effect of inactivating or deleting T cells. The mechanisms include a very rapid inhibition of TCR signal transduction (minutes) brought about by uncoupling of p56^{lck} from the early initiating events of TCR signaling, allowing the kinase to transmit direct inhibitory signals mediated via Dok-1 and SHIP-1 that may also reduce AKT activation. These effects combine with pronounced Fc-dependent effects which act with intermediate (ADCC-mediated deletion of CD4⁺ T cells) and slow kinetics (CD4 receptor down-modulation).

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