Development of a human monoclonal antibody for potential therapy of CD27-expressing lymphoma and leukemia

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Abstract:

**Purpose:** The TNF receptor superfamily member, CD27 is best known for its important role in T cell immunity, but is also recognized as a cell-surface marker on a number of B and T cell malignancies. In this report, we describe a novel human monoclonal antibody (mAb) specific for CD27 with properties that suggest a potential utility against malignancies that express CD27.

**Experimental Design:** The fully human mAb 1F5 was generated using human Ig transgenic mice and characterized by analytical and functional assays *in vitro*. Severe combined immunodeficiency (SCID) mice inoculated with human CD27-expressing lymphoma cells were administered 1F5 to investigate direct anti-tumor effects. A pilot study of 1F5 was conducted in non-human primates to assess toxicity.

**Results:** 1F5 binds with high affinity and specificity to human and macaque CD27, and competes with ligand binding. 1F5 activates T cells only in combination with T cell receptor stimulation, and does not induce proliferation of primary CD27-expressing tumor cells. 1F5 significantly enhanced the survival of SCID mice bearing Raji or Daudi tumors, which may be mediated through direct effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC). Importantly, administration of up to 10 mg/kg of 1F5 to cynomolgus monkeys was well tolerated without evidence of significant toxicity or depletion of circulating lymphocytes.

**Conclusions:** Collectively, the data suggest that the human mAb 1F5, which has recently entered clinical development under the name CDX-1127, may provide direct anti-tumor activity against CD27 expressing lymphoma or leukemia, independent of its potential to enhance immunity through its agonistic properties.
Translational Relevance

Monoclonal antibody (mAbs) based therapies have had a tremendous impact on the treatment of cancer, and in particular for lymphoma. The high expression of CD27, a T cell co-stimulatory molecule, on many types of lymphoma and leukemia prompted us to develop human anti-CD27 mAbs and determine their activity against lymphoma cells in vitro and in vivo. The anti-CD27 mAb 1F5 was effective in mediating antibody-dependent cellular cytotoxicity of human lymphoblastic cell lines and significantly reduced their growth when transplanted into immunodeficient mice. Although 1F5 could activate human T cells when combined with T cell receptor stimulation, 1F5 did not promote the survival or proliferation of freshly isolated lymphoma cells from patients. 1F5 was well tolerated and without significant depletion of lymphocytes in a pilot study with non-human primates. These studies have led to a clinical trial with this novel approach.

Introduction

Since the initial approval of rituximab in 1997, the continued success of monoclonal antibody (mAb) therapy in treating various types of lymphoma and leukemia has spurred considerable development of novel therapeutics including both naked antibody approaches and antibody-drug conjugates (1, 2). This success is thought to be driven in part by the accessibility of these types of tumors to antibodies and immune effector cells, as well as the number of tissue specific receptors that provide potential therapeutic targets. In addition to the targets with FDA approved antibodies, CD20 and CD52, there are multiple antibody based therapies targeting hematopoietic markers including, CD19,
CD22, CD30, CD38, CD40, CD45, CD70, CD74, and CD80 that are in clinical development as listed in clinicaltrials.gov. Importantly, although the side effect hypogammaglobulinemia has been observed with rituximab, the presence of the targeted receptors on both the malignant cells as well as the normal counterparts has not caused significant limitations to these approaches.

The TNF receptor superfamily member, CD27, is a tightly regulated co-stimulatory molecule activated by ligation through its unique counter-receptor, CD70 (3). In humans, CD27 is expressed on the surface of the majority of T cells, memory B cells and plasma cells, and some NK cells (4-6). The interaction of CD27 with CD70 plays an important role in the activation, proliferation, and survival of T cells; in clonal B cell expansion and germinal center formation; and in NK cell cytolytic activity (7-10). Recent studies show that modulation of this pathway by engineered expression of CD70 or agonist anti-CD27 antibodies can overcome tolerance and circumvent CD40-mediated signaling, leading to anti-tumor immunity in experimental systems (11-14). In addition, expression of CD70 on lymphoma and other experimental tumor cell lines has been shown to promote their rejection by both NK and T cell dependent mechanisms in immunocompetent mouse tumor models (15, 16), and CD27 activation is critical for the appropriate generation of anti-leukemia T cell response in B cell precursor acute lymphoblastic leukemia (17).

The expression of CD27 on various types of lymphomas and leukemias has been well documented (18-23). CD27 expression is present on most B cell malignancies at varying levels, and is also expressed by adult T-cell leukemia/lymphoma (18, 19, 24). An important observation has been the correlation of tumor burden in patients with acute and chronic B-cell malignancies and levels of a soluble form of the receptor, sCD27 that is
presumably released by enzymatic cleavage (18). Despite the abundance of CD27 on these tumors, we are not aware of any efforts to develop therapeutic anti-CD27 antibodies for potential use in patients with lymphoma or leukemia.

To determine if CD27 is a potential therapeutic target for antibody therapy of CD27-expressing tumors, we developed a fully human monoclonal antibody by use of human immunoglobulin expressing mice (25). In this study, we report the characterization of this human anti-CD27 antibody’s activity in vitro and in xenograft models with human lymphoma cell lines. In addition, we investigated the feasibility of this approach by administering the antibody to non-human primates. The human anti-CD27 antibody, 1F5, shows potent anti-tumor activity and does not result in direct T cell activation or significant depletion of CD27+ lymphocytes in vivo and represents a novel opportunity for testing in clinical trials.
Materials and Methods

Generation of CD27-specific human monoclonal antibodies

Human anti-CD27 monoclonal antibodies were generated by immunizing the HC2/KCo7 strain of HuMAb transgenic mice (26). The mice were immunized with recombinant human CD27 extracellular domain (R&D Systems, Minneapolis, MN). Immunization was carried on until mice reached human anti-CD27 titers >1:4000 (range 1:4000 – 1:64000) at which time spleens were collected for the preparation of hybridomas. Splenocytes were fused with P3x63Ag8.653 murine myeloma (CRL 1580, ATCC Manassas, VA) in the presence of polyethylene glycol (PEG, Sigma, St. Louis, MO). Fusion cells were selected in HAT medium (hypoxanthine, aminopterin, thymidine; Sigma, St. Louis, MO) and then screened for CD27- specific human IgG.

Antibody sequencing and expression

The VH and VL coding regions of the 1F5 anti-CD27 mAb were cloned and sequenced using the following primers; 5’RACE PCR: 5’- GTGCCAGGGGGAAGACCGATGGG for VH and 5’- GCAGGCACACAACAGAGGCAGTTCCATTC for VL. PCR product was sequenced and cloned into an expression vector. V(D)J segments usage and complementary determining regions (CDRs) were identified by alignment analysis using DNAPlot at the integrative germ-line V gene database (V-BASE2). The glutamine synthetase (GS) gene expression system (Lonza Biologics, Slough, UK) was used for production. A combined GS double-gene vector containing the heavy and light chains of 1F5 was introduced by electroporation using a Gene Pulse II (Bio-Rad, Hercules, CA) into CHOK1SV cells (Lonza Biologics). After selection in L-methionine sulphoximine...
(MSX, Fisher Scientific, Pittsburgh, PA), transfectomas were subcloned prior to expansion for production. The 1F5 mAb was purified by protein A column chromatography. Each batch of 1F5 was tested for endotoxin which never exceeded 1 EU/mL.

**Flow cytometry with lymphoma cell lines and PBMC**

1F5 or isotype control mAb was incubated with Raji, Ramos or Daudi cells (ATCC) at ambient temperature for 20 minutes. Cells were washed and cell-bound antibody was detected with a PE-labeled goat anti-human IgG Fc-specific antibody and analyzed on a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA). For competition assays, Daudi cells were incubated with recombinant human CD70-biotin (3 μg/ml) in the presence or absence of 1F5 or isotype control mAb at 50 μg/ml). Binding of CD70 was detected with streptavidin-PE.

For peripheral blood mononuclear cells (PBMC) experiments, whole blood was obtained from healthy human volunteers (Biological Specialties, Colmar, PA) or from rhesus or cynomolgus macaques and processed to PBMC by Ficoll density gradient centrifugation. FITC-labeled 1F5 or isotype control mAb was incubated with the PBMC’s and cell subset markers for 20 minutes at room temperature. Cells were washed and analyzed. Subset markers include CD3+ T cells, CD3+CD4+; CD8+ T cells, CD3+CD8+; B cells, CD19+CD20+; human NK cells, CD56+; monkey NK cells, CD3-CD8+CD16+.
CD27 ELISA

The CD27 ELISAs were performed using purified rhesus macaque CD27 (rhesus and cynomolgus are identical in the amino acid sequence of the extracellular domain) or human CD27. Briefly, we cloned the extracellular domain of CD27 from mRNA isolated from macaque or human PBMC by RT-PCR. The CD27 cDNA was introduced in a mammalian expression vector as an in-frame fusion protein with human kappa light chain for secretion and purification, and a FLAG-tag sequence was added at the C-terminus. The vectors were used for transient transfection of HEK 293 FreeStyle cells, and supernatants collected for purification by protein L chromatography. Purified macaque CD27 or human CD27 was captured to ELISA plates with anti-FLAG antibody, followed by incubation with anti-human CD27 mAb. A goat anti-human IgG Fc-HRP antibody and substrate Super Blue TMB were used for detection.

Samples were analyzed at OD$_{450}$ using a microtiter plate reader and IC$_{50}$ were derived by using software SoftMax Pro V5. Specificity of IF5 for CD27 was examined by ELISA using microtiter plates coated with other TNFR superfamily members, 4-1BB, CD40, Fas and TNFRSF1A (R&D Systems, Minneapolis, MN) and their respective antibodies for positive controls.

Biacore analysis

Binding affinity and binding kinetics IF5 was examined by surface plasmon resonance (SPR) analysis using a Biacore 2000 SPR instrument (Biacore AB, Uppsala, Sweden) according to the manufacturer’s guidelines. CD27/Fc (R&D Systems, Minneapolis, MN) was covalently linked to a Biacore CM5 sensor chip using standard amine coupling.
chemistry with an Amine Coupling Kit provided by Biacore. The 1F5 antibody was allowed to flow over the sensor chip at concentrations ranging from 0.4 to 200 nM and at a flow rate of 30 µl/minute for 180 seconds. A blank flowcell with no protein immobilized was used for background subtraction. The affinity and kinetic parameters, the association and dissociation curves were derived using the BiaEvaluation software (Biacore AB).

**Xenograft models**

Severe combined immune deficient mice CB.17 SCID mice (Taconic, Cambridge City, IN) were maintained in a specific pathogen-free (SPF) mouse facility. Raji cells (5 x 10^5) were inoculated by subcutaneous injection for local growth in the flank of mice. Animals were divided into 6-7 per group and treated by intraperitoneal injection with 1F5, isotype control or saline on day 5, 8, 12, 15, 19, and 22 after tumor challenge. Tumor growth was measured at regular intervals and tumor volume was calculated with modified ellipsoid formula (Length x width^2 ÷ 2). Mice were euthanized by pre-defined IACUC approved criteria. For the disseminated tumor model, Daudi cells (1 x 10^7) were injected intravenously into SCID mice. Groups of 10 mice were treated on day 5, 8, 12, 15, 19, and 22 after tumor challenge with 0.5 mg of 1F5 or isotype control. Survival was analyzed with Kaplan-Meier survival curve (MedCalc Statistical Software).

**Antibody dependent cell-mediated cytotoxicity (ADCC)**

Target cells (Ramos and Daudi cells) were loaded with Calcein AM (Life Technologies, Carlsbad, CA), washed and added to round-bottom microtiter wells. 1F5 and human IgG1
control antibodies were diluted in media and added to the wells of a round-bottom plate. Finally PBMC were added to yield effector:target ratio of 75:1. Cells were briefly pelleted in the plate and incubated for 4 hours at 37°C, 5%CO₂. Following the incubation, 75μl of supernatant from the wells were transferred into a clear bottom, black-walled plate. Fluorescence (Ex 485; Em 535) was recorded on a Perkin Elmer Victor X4 plate reader. Specific cytotoxicity (percentage) was determined using the following formula: (experimental lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis) x 100.

Proliferation and Cytokine induction

For proliferation studies, anti-CD3 mAb (OKT3) was added to the wells of a 96 well tissue culture plate with 1F5 mAb or huIgG1 isotype control in a final volume of 50μL. The antibodies were allowed to dry overnight in the well. In selected wells, the 1F5 mAb or huIgG1 isotype control was added in aqueous solution to the previously coated anti-CD3 well. After washing the wells with PBS, 100,000 CD3+ cells prepared from healthy donors were added to each well in a total of 200μl. The plates were incubated for 5 days at 37°C and 5%CO₂, and proliferation was measured with CellTiter Glo luminescent cell viability assay (Promega, Madison, WI). For intracellular cytokine analysis, anti-CD3 mAb (OKT3) was added to the wells of a 24 well tissue culture plate with 1F5 mAb or huIgG1 isotype control in a final volume of 200μL. The antibodies were allowed to dry overnight in the well. After washing the wells with PBS, 1.5 x 10⁶ CD3+ cells prepared from healthy donors were added to each well in a total of 1mL. The plates were incubated for 2 days at 37°C and 5%CO₂. The cells were harvested and stained with the
following labeled antibodies for surface expression: CD4 PerCP-Cy5.5, CD8 APC-H7, CD45RO FITC, and appropriate isotype controls. The cells were fixed with 1X Becton Dickinson Lysing Buffer for 10 minutes, then washed and resuspended in 1X Becton Dickinson Perm 2 Solution for an additional 10 minutes. After permeabilization, the cells were stained for intracellular markers as follows: IFN-γ APC, TNF-α PE, CD69 V450, and appropriate isotype controls. The samples were analyzed on a Becton Dickinson FACSCanto II flow cytometer within 24 hours of staining. Using appropriate T cell subset gating strategies, the percentage of activated cells positive for the cytokines was determined.

**Proliferation with primary human tumor cells**

Patients providing written informed consent in accordance with the Declaration of Helsinki were eligible for this study if they had a tissue biopsy that on pathologic review showed B-cell lymphoma and adequate tissue to perform the experiments. The biopsy specimens were reviewed and classified using the World Health Organization (WHO) lymphoma classification. The use of human tissue samples for this study was approved by the Institutional Review Board of the Mayo Clinic/Mayo Foundation. Fresh biopsy specimens were dissociated manually and the cells filtered through a wire mesh filter. The cell suspension was purified using a Ficoll gradient. CD19+ cells were isolated for by positive selection using magnetic beads and a Robosep (Stem Cell Technologies, Vancouver, BC). For flow cytometry, cells were incubated with IgG isotype control or CD27 antibody (eBioscience, San Diego, CA) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). For proliferation studies, the antibodies
were added in triplicate to wells of a 96 well tissue culture plate in a final volume of 100 ul. The antibody was allowed to dry overnight in the well. After washing the wells with PBS, 100,000 CD19+ tumor cells were added to each well in a total of 200 ul. The cells were incubated for 72 hours at 37° in 5% CO₂. At 48h, 20ul of [³H]thymidine (1uCi) was added to each well. Cells were harvested onto glass fiber filters using a FilterMate Harvester (Perkin Elmer, Cambridge, MA) and radioactive incorporation was counted using a MicroBeta TriLux LSC Counter (Perkin Elmer).

**1F5 administration to cynomolgus macaques**

Three male monkeys were treated with 1F5 mAb at Toxikon (Bedford, MA) under an IACUC-approved protocol. The animals received a single intravenous infusion of 1 mg/kg, 3 mg/kg or 10 mg/kg of 1F5 mAb. Parameters evaluated for the assessment of toxicity included body weight, clinical observations, physical exams, hematology, blood chemistry, urinalysis, and body temperature. Serum samples were collected at baseline, and at 4, 24 and 48 hours post dosing for analysis of cytokine levels using ELISA-based kits for TNF-α, IL-6, and IL-1β (Bender Medsystems, eBioscience, Inc. San Diego, CA). Whole blood was collected and shipped overnight for flow cytometry analysis. The following antibodies were used to stain the cell populations and were obtained from BD Biosciences: CD3 V450, CD4 APC, CD8 PerCP-Cy5.5, CD16 APC, CD20 APC, CD95 PE, CD69 FITC, CD25 PerCP-Cy5.5, and HLA-DR FITC. In addition, CD127 PE obtained from Beckman Coulter (Indianapolis, IN) and goat anti-human IgG FITC from Bethyl Laboratories (Montgomery, TX) was used. Analysis of the samples was performed on a Becton Dickinson FACSCanto II flow cytometer. All of the lymphocyte
subset analysis, with the exception of B cells, utilized whole blood staining on the day it was received. B cells were stained using purified leukocytes after red cell lysis with KHCO₃. The following cell types were defined by the gating: CD3+ cells, CD4+ cells, CD8+ cells, B cells (CD20+), memory B cells (CD20+, CD95+), naïve B cells (CD20+, CD95-), NK cells (CD3-, CD8+, CD16+) and regulatory T cells (CD4+, CD25+, CD127dim). Lymphocyte and monocyte populations were defined by side scatter and size.

**Statistical analyses**

Statistical analysis on the xenograft experiments were performed using Wilcoxon two-sample test analysis and the Kaplan-Meier MedCalc software program.
Results

Development of human antibodies specific for CD27

Transgenic mice expressing human immunoglobulin genes have been useful for generating human antibodies to important therapeutic targets through standard hybridoma technology (24). To generate human antibodies specific for CD27, HuMAb mice were immunized with recombinant human CD27-Ig fusion protein. Spleen cells derived from immunized animals were fused with the murine myeloma line P3X63Ag8.653, and subsequent hybridomas were screened for human IgGκ that bound to CD27 by ELISA or to CD27+ lymphoma cell lines. Selected hybridomas were used for cloning and sequencing of the variable heavy and light chain genes, which were subsequently introduced into Ig expression vectors for production by transient or stable transfection in CHO cells.

Human mAb 1F5 binding characteristics

The human anti-CD27 IgG1κ antibody 1F5 was confirmed to contain somatic mutations in the complementarity determining regions of both the heavy and light chains, and was purified for further characterization. The 1F5 mAb has strong reactivity with three human Burkitt's lymphoma cell lines, Raji, Daudi, and Ramos (Figure 1A), and efficiently blocked the interaction between CD70 and CD27, suggesting an epitope specificity in the ligand binding domain (Figure 1B). In addition, Biacore analysis demonstrated the 1F5 mAb has sub-nanomolar equilibrium dissociation constants for binding to human CD27. The binding affinity and binding kinetics of CDX-1127 were
performed by surface plasmon resonance analysis using a CD27 coated CM5 sensor chip. The mean values from 2 separate runs were $k_a = 3.07 \times 10^5$ (1/Ms), $k_d = 5.72 \times 10^{-5}$ (1/s), $K_a = 5.37 \times 10^9$ (1/M) and $K_d = 1.86 \times 10^{-10}$ (M). We also confirmed that 1F5 does not bind to related members of the TNFR superfamily, 4-1BB, CD40, Fas and TNFRSF1A (data not shown).

The selection of 1F5 mAb was partly based on cross-reactivity to macaque CD27 that could provide a critical model for establishing the safety and functional characteristics of antibodies with potential clinical utility. For this purpose, we cloned and expressed the extracellular domain of CD27 from human, rhesus macaque and cynomolgus macaque PBMCs. The amino acid sequence for rhesus and cynomolgus macaque was identical and therefore interchangeable for these species. An ELISA assay demonstrating similar binding of 1F5 to CD27 from human and macaques is shown in Figure 1C. The concentration yielding 50% of maximum binding (IC$_{50}$) of 1F5 was 0.059 µg/ml, and 0.12 µg/ml for human and macaque CD27, respectively. To further establish the relevance of macaques as a model for testing 1F5, we compared the binding pattern of 1F5 to peripheral blood lymphocytes from 6 individual humans and monkeys (Table 1). Overall, similar binding was observed. The greatest disparity between human and monkey 1F5 binding was evident in the NK cells, with the monkeys showing a much higher percentage of CD27 expressing cells than humans. 1F5 binding to human T cells resulted in greater cell-associated fluorescence compared to monkey T cells indicating a higher level of CD27 expression. The B cell staining was very comparable between the species. These data were confirmed with a commercial anti-CD27 mAb (clone MT-271)
and thus appear to reflect real differences and similarities in CD27 expression patterns between the two species (data not shown).

1F5 mAb inhibits the growth of transplantable human lymphoma cells in SCID mice

The selection of the 1F5 mAb was also based on its ability to inhibit the growth of CD27-expressing tumors in xenograft models. To assess the anti-tumor activity of 1F5, we administered the antibody to immunodeficient SCID mice after inoculation with human Burkitt’s lymphoma derived Raji or Daudi cells. Relatively low doses of antibody were effective in significantly retarding the growth of the subcutaneously implanted Raji cells and improved survival (Figure 2A and 2B). Interestingly, the trend toward lower efficacy at higher 1F5 dose was consistently observed in Raji xenograft model. However, this was not observed in other tumor xenograft models, which showed similar or better activity at the 0.3 mg dose (data not shown). Similarly, 1F5 had significant effect on the survival of mice in a disseminated tumor model using intravenous inoculation of Daudi cells (Figure 2C).

Effector functions of 1F5 mAb

The anti-tumor effects of 1F5 in the SCID xenograft models suggest that 1F5 can mediate effector functions such as ADCC or complement dependent cytotoxicity (CDC), and/or might lead to direct inhibition of tumor cell growth through induction of apoptosis. Although 1F5 mAb is an IgG1 mAb, an isotype capable of efficient complement fixation, we were unable to demonstrate significant CDC activity against the lymphoblastoid cell
lines, Daudi, or Ramos using a variety of complement sources (data not shown). Similarly, 1F5 did not appear to have significant effects on the growth or apoptosis of these cell lines in vitro (data not shown). However, we observed significant ADCC activity of 1F5 using human PBMC effector cells and Ramos or Daudi tumor cells (Figure 2D). This suggests that ADCC may be the dominant mechanism for the activity of 1F5 in the xenograft models.

**Effect of 1F5 on human lymphocytes in vitro**

We used in vitro assays with human PBMC or purified T cells to address the potential concern that the engaging CD27 with 1F5 on human lymphocytes could lead to non-specific proliferation and cytokine release. Consistent with the known biology of CD27 we demonstrated the 1F5 mAb does not lead to direct activation of lymphocytes in the absence of additional signals. 1F5 was unable to activate human lymphocytes to divide (Figure 3A, left panel) even under conditions that favor maximum cross-linking of the receptor (coated wells). However, combining 1F5 with suboptimal levels of T cell receptor stimulation using anti-CD3 mAb (OKT3) was shown to enhance proliferation of human T cells (Figure 3A, right panel). The 1F5 mediated proliferation required the antibody to be immobilized, suggesting the requirement for cross-linking. Similarly, the combination of 1F5 and OKT3 also led to significant IFN-γ and TNF-α from both CD4 and CD8 T cells using intracellular cytokine analysis (Figure 3B). These data are consistent with the requirement for a second signal via the T cell receptor for CD27 mediated signaling to induce T cell activation (27, 28), and confirm the agonist properties of 1F5 mAb.
Effect of 1F5 on primary lymphoma biopsy samples

Although 1F5 clearly inhibited the growth of lymphoma cell lines when transplanted into SCID mice, we investigated the potential of 1F5 to enhance the proliferation of primary lymphoma biopsy samples. Fresh primary tumor biopsy samples were collected from patients with various hematological malignancies and sorted to separate CD19 expressing cells, the vast majority of which are malignant cells. Patients with marginal zone, mantle cell and follicular lymphomas had particularly high levels of CD27 expression (Figure 4A). The isolated tumor cells were exposed to varying concentrations of 1F5 or isotype controls that were dry-coated to wells to provide maximum cross-linking (Figure 4B). After 72 hours incubation, a dose-dependent decrease in proliferation was observed in all of the specimens, and this effect was similar for both 1F5 and the isotype control suggesting the inhibitory effect may be the result of Fc receptor binding. The mechanism for this Fc receptor-mediated effect is unknown, although it may be a result of signaling through the inhibitory Fc receptor IIB (FcRIIB), which is the predominant Fc receptor expressed in the B cell lineage, and targeting B lymphomas with an antibody to FcRIIB has shown anti-tumor effects in xenograft models with human lymphoblastic cells (29). Importantly, the 1F5 mAb did not enhance the proliferation of the CD19+ tumor cells.

Effect of 1F5 on circulating lymphocytes in monkeys

A preliminary in vivo study was conducted to assess the potential of 1F5 to cause lymphocyte activation or depletion that may result in safety concerns. Three male
cynomolgus macaques (7.65-8.95 kg) were treated with 1, 3, or 10 mg/kg 1F5 mAb administered i.v. over approximately 30 minutes, and followed for 29 days. All doses were well tolerated with no findings during physical exams (days 1 and 3) and none of the animals exhibited any signs of toxicity over the course of the study including body temperature and weight. In addition, measurement for the serum cytokine levels of TNF-α, IL-6, and IL-1β at 4, 24 and 48 hours post dosing was below detection in all cases. The effect of 1F5 on circulating lymphocyte populations was monitored by flow cytometry on blood samples drawn on day 1 (pre-dose) and on days 2, 3, 8 and 29. Overall, there were no significant long-term changes in major lymphocyte sub-populations, although transient changes were observed in some cases (Figure 5A). While the overall percentage of CD3+ cells remained relatively unchanged, 1 day after dosing there is a 15-20% increase in CD4+ cells and a corresponding 20-30% reduction in CD8+ T cells. These changes are very transient as the percentages of CD4+ and CD8+ T cells are similar to baseline 2 days after treatment. Interestingly, the percentage of Treg cells decreased by 25-50% in the animals treated with the higher doses (3 or 10 mg/kg) and remained lower at day 29. The percentage of CD20+ B cells showed modest increases over the course of the study, and the memory B cell population (CD27+) remained near baseline levels or showed some increase. The most remarkable finding was a 60-90% decrease in circulating NK cells at day 1 and 2, which recovered by day 8 in 2 of 3 animals and in all animals at day 29.

There were no consistent changes in activation markers (CD69 or HLA-DR) on lymphocytes, with the exception of NK cells at the highest dose level, showing moderate up-regulation of HLA-DR (data not shown). We also monitored the presence of 1F5 on
circulating lymphocytes by staining cells with anti-human IgG (Figure 5B). Even at the lowest dose of 1 mg/kg, the lymphocytes and memory B cells were saturated with 1F5 throughout the 29 day study. This was not surprising as low levels of circulating antibody could still be detected in this animal at day 29 (0.25 μg/ml).

Discussion

Although the expression of CD27 on various lymphomas and leukemias is well documented, we are not aware of reports that address the potential of CD27 as a direct therapeutic mAb target. Here we have described the characterization of a new human anti-CD27 mAb, 1F5, which was selected from a panel of fully human anti-CD27 antibodies generated using human Ig transgenic mice. We selected 1F5 mAb due to its high affinity for CD27, cross-reactivity with rhesus and cynomolgus macaque CD27, and activity in several in vivo models. In addition, 1F5 was shown in competition assays to bind at the ligand binding domain of CD27, which may improve its ability to mimic the ligand mediated receptor cross-linking.

The therapeutic potential of 1F5 was demonstrated in xenograft models using human lymphoblastoid cell lines grown in SCID mice. Mice treated over the range of 33-300 μg 1F5 mAb per dose x 6 (approximately 1.5 – 15 mg/kg) showed a significant reduction in subcutaneous Raji tumor cell growth and complete eradication in a small proportion of the mice. Importantly, the 1F5 treatment was also effective in improving the survival of mice using a disseminated model of i.v. administered Daudi lymphoma cells. In these models, the antibody can only react with CD27 on the human tumor cells, due to the lack
of any binding by 1F5 to mouse CD27 (data not shown), suggesting that the anti-tumor activity is based on effector mechanisms such as ADCC, CDC, or growth inhibition/apoptosis via CD27 signaling in tumor cells. In addition, we have observed significant ADCC with the 1F5 mAb using several human lymphoma cell lines. In contrast, we did not observe significant CDC, apoptosis or direct growth inhibition under the conditions of the experiments we performed.

Recent studies have documented the potential for anti-CD27 antibodies in mouse lymphoma models (11, 13, 14). Interestingly, in these studies, the primary mechanism of anti-tumor activity was through indirect enhancement of the immune responses against the tumor, and did not require CD27 expression by the lymphoma cells. In addition, Sakanishi, T. and Yagita, H. demonstrated that non-depleting anti-CD27 mAbs were most effective in eradicating the tumors, suggesting the antibody therapy did not rely on direct elimination of target-expressing cells as observed with anti-CD20 mAb therapy (14). These data suggest agonist anti-CD27 mAbs may have potential for treatment of lymphoma and leukemia, as well as other cancers through immune activation mechanism providing T cell co-stimulatory and survival signals. To test for agonist properties of 1F5, we showed that suboptimal levels of T cell receptor stimulation using anti-CD3 mAb (OKT3) combined with the 1F5 mAb enhanced proliferation of human T cells. Therefore, the 1F5 mAb may additionally contribute to anti-lymphoma and leukemia responses through indirect immune activation mechanisms. Experiments with 1F5 mAb performed in human CD27 transgenic mice further support this mechanism (manuscript in preparation).
The effector functions and agonistic properties of 1F5 prompted concern that the antibody may result in depletion of T cells and/or non-specific activation that could limit the therapeutic utility and generate unwanted side-effects. We demonstrated the relevance of cynomolgus macaques for testing 1F5 by showing comparable binding and expression between human and macaque CD27. Cynomolgus macaques treated with a single dose of 1, 3, or 10 mg/kg 1F5 had no significant noted pathologic changes over a 29 day monitoring period. Further, the 1F5 treatment did not result in significant long term depletion of peripheral blood lymphocytes. The inability to deplete normal circulating CD27+ cells may seem a contradiction to the anti-tumor activity of 1F5 in the xenograft models, however, this may be the result of the very high expression of CD27 on the lymphoblastic cell lines, and differential signaling mechanisms occurring in rapidly dividing malignant cells. These data, show 1F5 is well tolerated in non-human primates, however, we cannot exclude the possibility of potential effects beyond the treatment period or with multiple dosing. Taken together with the in vitro data demonstrating the lack of T cell activation without TCR stimulation, the data suggest that 1F5 mAb should have a good safety profile in humans.

The agonistic property of 1F5 further raises the theoretical concern of enhancing the proliferation of CD27-expressing tumors. The xenograft models with human tumor cell lines clearly show that the 1F5 inhibits tumor growth, but these studies are limited by the use of culture adapted tumor cell lines. Importantly, we also demonstrate the 1F5 mAb does not enhance the proliferation of CD27-expressing, freshly isolated, hematologic tumor cells even under conditions that favor maximal cross-linking. Collectively, these data suggest that the novel human anti-CD27 mAb, 1F5, may have therapeutic activity.
against CD27-expressing hematological malignancies without undue side-effects resulting from lymphocyte depletion or overt activation. Based on these studies, the 1F5 mAb (CDX-1127) is now undergoing a phase 1 study in patients with hematological malignancies.

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References


Figure 1. 1F5 mAb binding to lymphoma cell lines and recombinant human and macaque CD27. Panel A; The lymphoblastoid cell lines, Raji, Ramos, and Daudi were incubated with 10 μg/ml 1F5 or isotype control and detected with a PE-labeled goat anti-human IgG Fc-specific antibody. Cell associated fluorescence was determined by flow cytometry. The plotted histograms show human IgG1 isotype (filled histogram) and 1F5 (open histogram). Panel B; Daudi cells were incubated with 3 μg/ml CD70-biotin alone (top panel) or with CD70-biotin and 50 μg/ml isotype control mAb(middle panel) or with CD70-biotin and 50 μg/ml 1F5 mAb (lower panel). Binding of CD70 was detected with streptavidin-PE. Panel C; The extracellular domain of monkey CD27 (squares) and human CD27 (circles) were produced with a FLAG tag and captured to ELISA plates with anti-FLAG antibody, followed by incubation with various concentrations of 1F5 mAb. Control wells had irrelevant protein (DEC-205-FLAG) captured. A goat anti-human IgG Fc-HRP antibody and substrate Super Blue TMB were used for detection.

Figure 2. In vivo and in vitro anti-tumor activity of 1F5 against lymphoblastoid cell lines. Panel A; Groups of 6-7 SCID mice were injected s.c. with 5 x 10^5 Raji cells and then administered 6 doses (indicated by arrows) of 1F5 or isotype control by i.p. injection at the doses indicated. Animals were followed until euthanized based on IACUC defined criteria. Statistics were performed on day 33 results, all 3 treatment groups were significantly different than controls with P <0.01 or lower using Wilcoxon two-sample Research.
test analysis. Panel B; Kaplan-Meier curve of the experiment shown in A. C, Kaplan-Meier analysis of groups of 10 animals that were injected i.v. with $10^7$ Daudi cells and then administered 6 doses (indicated by arrows) of 1F5 or isotype control by i.p. injection at the dose indicated. The p value for the 1F5 treated animals against controls was p=0.0056. Graphs are representative of 2 or more experiments. Panel D; Ramos or Daudi cells were used as target cells for ADCC assays as described in Materials and Methods. PBMC from healthy donors were used as effector cells. Target and effector cells were combined at an effector:target ratio of 75:1. Lysis of targets is shown for 1F5 (solid line) and control human IgG1 (dashed line) antibodies.

Figure 3. CDX-1127 Enhances T Cell Proliferation Induced by T Cell Receptor Stimulation. CD3+ T cells were purified from PBMC and seeded into microtiter wells that were previously coated by air drying antibodies in selected wells as described in Materials and Methods. Panel A; Proliferation was assessed using a CellTiter-Glo luminescent cell viability assay (Promega) per manufacturers protocol after 5 days incubation. As control, some wells received 1F5 or human IgG in soluble form rather than coated to the plate. The stimulation index was calculated by dividing the sample value with the value from media controls and is expressed as the mean ± SE. Panel B; Cells were harvested after 2 days incubation with plate coated antibodies (10 μg/ml) or media control and stained for intracellular expression of IFN-γ or TNF-α.
Figure 4. Effect of 1F5 on the proliferation of CD27-expressing primary human tumor biopsy samples. Biopsy specimens were dissociated manually and the CD19+ cells were isolated by positive selection using magnetic beads and a RoboSep (Stem Cell Technologies, Vancouver, BC). Panel A; Cells were incubated with IgG isotype control (shaded) or CD27 antibody (solid line) and analyzed on a FACSCalibur flow cytometer. Histograms from representative biopsy specimens are shown. Panel B; IF5 or IgG control were added in triplicate to wells of a 96 well tissue culture plate allowed to dry overnight in the well. 100,000 CD19+ tumor cells were added to each well in a total of 200µl. The cells were incubated for 72h and proliferation was assessed by incorporation of [³H]thymidine.

Figure 5. Effect of 1F5 on circulating lymphocytes in cynomolgus macaques. Blood was drawn at various time points from cynomolgus macaques treated with 1F5 mAb (Pre-dose- just prior to administration, 2 hours post dosing on day 1, day 2, 8, and 29). Panel A; Lymphocytes were stained with subset markers and the % positive cells plotted vs time for each animal treated at the different doses. Black circles, 1 mg/kg ; black squares, 3 mg/kg; black triangles, 10 mg/kg. Panel B; Total lymphocytes (based on side and forward scatter size), memory B cells (CD20+ and CD95 bright), and monocytes (based on side and forward scatter size) were stained with anti-human IgG specific secondary antibody (bold line) and compared to unstained controls (shaded histogram) using flow cytometry analysis.
Figure 1
Table 1. 1F5 mAb binding to human and monkey peripheral blood cells

<table>
<thead>
<tr>
<th>Analysis</th>
<th>CD4+ T cells</th>
<th>CD8+ T cells</th>
<th>B cells (CD20+)</th>
<th>NK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>human</td>
<td>monkey</td>
<td>human</td>
<td>monkey</td>
</tr>
<tr>
<td>% CD27+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87 ± 3</td>
<td>76 ± 4</td>
<td>73 ± 6</td>
<td>84 ± 5</td>
</tr>
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<td>MFI&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2018 ± 197</td>
<td>612 ± 49</td>
<td>1990 ± 250</td>
<td>763 ± 79</td>
</tr>
</tbody>
</table>

<sup>a</sup>PBMCs were purified from blood of six cynomolgus macaques and 6 human donors for staining with lymphocyte markers, anti CD-27 mAbs (1F5 ) and isotype controls. The following markers were used for each cell type: CD4+ T cells, CD3+CD4+; CD8+ T cells, CD3+CD8+; B cells, CD19+CD20+; human NK cells, CD56+, monkey NK cells,
Figure 2
Figure 4

(A) Proliferation, CPM

- 0
- 500
- 1000
- 1500

- Media
- IgG 0.1 μg/ml
- IgG 1.0 μg/ml
- IgG 5.0 μg/ml
- 1F5 0.1 μg/ml
- 1F5 1.0 μg/ml
- 1F5 5.0 μg/ml

(B) 

- Follicular
- Marginal Zone
- Mantle Cell
Figure 5
Development of a human monoclonal antibody for potential therapy of CD27-expressing lymphoma and leukemia

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