Generation and Characterization of Fully Human Monoclonal Antibodies Against Human Orai1 for Autoimmune Disease

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ABSTRACT

Calcium entry into T cells following antigen stimulation is crucial for nuclear factor of activated T cells (NFAT)–mediated T cell activation. The movement of calcium is mediated by calcium release–activated calcium (CRAC) channels. There are two key components of this channel: Orai1 is the pore-forming subunit located in the plasma membrane, and stromal interaction molecule 1 (STIM1) functions as a Ca\(^{2+}\) sensor in the endoplasmic reticulum. A subset of human patients carry mutations in either STIM1 or Orai1 that affect protein function or expression, resulting in defective store-operated Ca\(^{2+}\) influx and CRAC channel function, and impaired T cell activation. These patients suffer from a hereditary form of severe combined immune deficiency syndrome, highlighting the importance of the CRAC channel for T lymphocyte function in humans. Since autoreactive T cells play an important role in the development of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and organ transplantation, Orai1 becomes an attractive therapeutic target for ameliorating autoimmune disease. We developed a novel approach to inhibiting CRAC function by generating high-affinity fully human monoclonal antibodies to human Orai1. These antibodies inhibited \(I_{\text{CRAC}}\) current, store-operated Ca\(^{2+}\) influx, NFAT transcription, and cytokine release. These fully human antibodies to human Orai1 may represent a novel therapeutic approach for the treatment of autoimmune disease.

Introduction

Intracellular calcium serves as a second messenger important in the regulation of gene expression, cell differentiation, cytokine secretion, and calcium homeostasis (Putney, 1986; Parekh and Putney, 2005). Store-operated calcium influx is a process that controls diverse functions such as, but not limited to, refilling of intracellular Ca\(^{2+}\) stores (Putney and Bird, 1993), activation of enzymatic activity (Fagan, et al., 2000), gene transcription (Lewis, 2001), cell proliferation (Nunez et al., 2006), and release of cytokines (Winslow et al., 2003). In the immune system, store-operated calcium influx is primarily mediated by calcium release–activated calcium channels (CRAC) (Hoth and Penner, 1993; Partiseti et al., 1994; Lewis, 2001; Feske et al., 2003, 2005; Hogan et al., 2003; Gallo et al., 2006; Feske, 2007). The molecular components of the CRAC channel have been identified. The channel consists of two essential proteins: stromal interaction molecule 1 (STIM1), which is localized in the endoplasmic reticulum (ER) and forms the Ca\(^{2+}\) store sensor (Liou et al., 2005; Roos et al., 2005), and Orai1, the pore-forming subunit (also known as calcium release–activated calcium modulator 1 or transmembrane protein 142A), which is located in the plasma membrane (Prakriya et al., 2006; Vig et al., 2006; Zhang et al., 2006).

In a T cell–mediated immune response, antigen recognition by T lymphocytes triggers phospholipase C\(^\gamma\) and generates inositol-1,4,5-triphosphate, resulting in the release of Ca\(^{2+}\) from the ER. Depletion of ER Ca\(^{2+}\) stores activates STIM1 and subsequently CRAC channels, resulting in Ca\(^{2+}\) influx across the plasma membrane. Sustained calcium influx, a hallmark of CRAC activity, leads to nuclear factor of activated T cells (NFAT) dephosphorylation by the calmodulin-dependent protein phosphatase calcineurin and subsequent NFAT translocation to the nucleus (Okamura et al., 2000). In the nucleus, NFAT activates the transcription of a variety of genes encoding for cytokines such as interleukin-2 (IL-2) and interferon-\(\gamma\) (IFN-\(\gamma\)) that are crucial for T cell activation (Feske et al., 2003).

A subset of patients with hereditary severe combined immune deficiency exhibit defective CRAC channel function

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ABBREVIATIONS: Ab, antibody; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid; Ch., chimeric; ConA, concanavalin A; CRAC, calcium release–activated calcium channel; ER, endoplasmic reticulum; ECL1, extracellular loop 1; ECL2, extracellular loop 2; FACs, fluorescence-activated cell sorting; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; HEK-293, human embryonic kidney 293s; hOrai1, human Orai1; hSTIM1, human STIM1; IFN-\(\gamma\), interferon-\(\gamma\); IL, interleukin; KinExA, Kinetic Exclusion Assay; mAb, monoclonal antibody; mOrai1, mouse Orai1; MSD, Meso Scale Discovery; NFAT, nuclear factor of activated T cells; PCR, polymerase chain reaction; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; POC, percentage of control; SNP, single-nucleotide polymorphism; STIM1, stromal interaction molecule 1; TG, thapsigargin; TM, transmembrane; YFP, yellow fluorescent protein.
due to various mutations in Orai1 (Feske et al., 2001, 2005, 2006, 2010; McCarl et al., 2009). All four identified mutations in Orai1 abolish CRAC channel currents and impair store-operated calcium entry in T cells. The clinical syndrome of these patients is limited and characterized by immunodeficiency, congenital myopathy, and anhydrotic ectodermal dysplasia with a defect in dental enamel calcification (McCarl et al., 2009; Feske, 2010). The CRAC channel has therefore attracted considerable attention as a therapeutic target for development of novel treatments for autoimmune disease, organ transplantation, and chronic inflammation.

Intense drug discovery efforts have been focused on the development of small-molecule antagonists of the CRAC channel. Several potent compounds have been described which inhibit CRAC channel current, prevent inflammatory mediator release in T lymphocytes, and exhibit efficacy in animal models of autoimmune disease (Ishikawa et al., 2003; Zitt et al., 2004; Yonetoku et al., 2006a,b, 2008; Yoshino et al., 2007). However, none of the reported small-molecule antagonists has been successfully moved into human clinical trials. We took a novel approach and generated high-affinity fully human monoclonal antibodies (mAbs) against human Orai1 (hOrai1) as an alternative to small-molecule antagonists of the channel. In this study, we report the binding properties and epitope of several mAbs to human Orai1. In addition, we demonstrate that the mAbs are functionally active and suppress thapsigargin (TG) and phorbol 12-myristate 13-acetate (PMA) + ionomycin–induced cytokine secretion in 50% human whole blood. Furthermore, we show that these fully human anti-hOrai1 mAbs reduce I_{CRAC} currents in a recombinant stable expression system, decrease Ca\(_{\text{2+}}\) influx and cytokine secretion in Jurkat cells, and attenuate NFAT transcriptional activity. Our results validate our monoclonal antibody strategy for targeting diverse classes of ion channels selectively (Naylor and Beech, 2009), and further support the concept of antagonizing CRAC as a potential therapeutic approach for autoimmune disease.

Materials and Methods

Establishment of hOrai1, hOrai1/human STIM1, hOrai1/YFP-human STIM1, hOrai1/YFP-human STIM1/NFAT-Luc, and Jurkat/AQN Recombinant Cell Lines. Recombinant human Orai1, human STIM1 (hSTIM1), and yellow fluorescent protein (YFP)-hSTIM1 cDNAs were cloned into mammalian expression vectors containing selectable markers. After transfection and appropriate drug selections, U2OS cells and CHO-AM1 (AM1) cells stably expressing hOrai1 were sorted by fluorescence-activated cell sorting (FACS) for hOrai1 using in-house-generated mouse anti-hOrai1 mAb. The AM1/hOrai1 cell line was subsequently transfected with YFP-hSTIM1 cDNA and sorted for YFP to create an AM1/hOrai1/YFP-hSTIM1 stable cell line. The human embryonic kidney 293 (HEK-293/hOrai1/hSTIM1) stable cell line was generated by cotransfection of hOrai1 and hSTIM1 cDNAs. Single-cell-derived stable functional cell lines were screened by both fluorescence imaging plate reader–based calcium influx and electrophysiological assays. HEK-293/hOrai1/YFP-hSTIM1 stable cells were developed by cotransfection of hOrai1 and YFP-hSTIM1 cDNAs. After selection, the pools were sorted by FACS for YFP and then subcloned, and clones were evaluated using Indo-1 AM (Life Technologies, Carlsbad, CA) ratiometric Ca\(_{\text{2+}}\) flux assay to create HEK-293/hOrai1/YFP-hSTIM1. Subsequently, the cell line was transfected with pGL4.30 (luc2P/NFAT-RE/Hygro) from Promega using Fugene HD (Promega Corporation, Madison, WI). The subclones were evaluated based on NFAT-luc activity resulting in the HEK-293 T/hOrai1/YFP-hSTIM1-NFAT-Luc cell line. JurkatAQN (Aequorin) stable cells were developed by transfection of Jurkat cells with plasmids encoding mitochondria-targeted aequorin using Amaxa Nucleofector (Lonza, Allendale, NJ). After selection, single-cell-derived stable functional cell lines were evaluated using an aequorin-based calcium assay to generate JurkatAQN cells.

Construction of hOrai1(S218G) and hOrai1(N223S) Variants. hOrai1-mouse Orai1 Extracellular Loop 2 Chimera Mutants, and mOrai1-hourOrai1 Extracellular Loop 2 Chimeric Mutants. Human Orai1 single-nucleotide polymorphism (SNP) variants, S218G and N223G, were generated by a site-directed mutagenesis polymerase chain reaction (PCR) using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) on the wild-type hOrai1 cDNA. Chimeric hOrai1-mouse Orai1 (mOrai1) extracellular loop 2 (ECL2) was generated by a two-part PCR strategy using the full-length human Orai1 and mouse Orai1 as templates. The resulting 5' fragment of the hOrai1-mOrai1 ECL2 and the 3' fragment of the hOrai1-mOrai1 ECL2 were digested and ligated into a mammalian expression vector. Chimeric mOrai1-hourOrai1 ECL2 was created using one forward primer and seven reverse primers in seven successive rounds of PCR amplification using full-length mOrai1 as a template in a standard PCR reaction. The resulting mOrai1-hourOrai1 fragment was digested and ligated into a mammalian expression vector. The hOrai1-mOrai1 ECL2 chimera mutants and mOrai1-hourOrai1 ECL2 chimera mutants were generated by site-directed mutagenesis using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacturer's instructions using chimeric hOrai1-mOrai1 ECL2 and chimeric mOrai1-hourOrai1 ECL2 as templates, respectively. All constructs were confirmed by sequencing.

Transient Expression. One day prior to transfection, HEK-293 cells were plated at 3.5 × 10^6 cells/dish in 10 ml of growth medium onto 100-mm tissue culture dishes (Corning Incorporated, Corning, NY). For one 100-mm dish, 10 μg of DNA was diluted in 460 μl of Opti-MEM (Life Technologies), mixed gently, and incubated at room temperature for 5 minutes. Then, 40 μl of FuGene HD (Promega Corporation) transfection reagent was added to the mixture, mixed gently, and incubated at room temperature for 20 minutes. The transfection mixture was added drop-wise onto the cells, and the dish was gently swirled to ensure uniform distribution of the complex. Cells from the HEK-293 transient transfection system were harvested at 48 hours post-transfection.

FACS Binding Analysis. Cells were washed once with ice-cold D-PBS (Dulbecco’s phosphate-buffered saline) (Life Technologies) and resuspended in ice-cold FACS buffer (1X D-PBS + 2% goat serum). Then 2 × 10^6 cells in 100 μl were stained per antibody combination. All antibody incubation steps were performed on ice for 1 hour. Cells were first incubated with 1 μg of unlabeled human anti-hOrai1 mAb, followed by a wash with 200 μl of FACS buffer. Next, the unlabeled antibody was detected using goat F(ab’2) anti-human IgG-phycocerythin (SouthernBiotech, Birmingham, AL) followed by a wash with 200 μl of cold FACS buffer before flow cytometry analysis. Binding to Orai1 on the surface of T cells was assessed in human blood. Human whole blood was obtained from healthy, nonmedicated donors in a Na-heparin vacutainer. Blood was incubated with V450 anti-CD3 (clone UCHT1) (BD Biosciences, San Jose, CA) and 1 μg of Alexa Fluor 647–labeled human anti-hOrai1 or anti-KHL for 30 minutes at room temperature. Pharm Lyse (BD Biosciences) was added to the blood and incubated for 15 minutes at room temperature to lyse red blood cells. The samples were then centrifuged at 1 400 g for 5 minutes, and supernatants were discarded and washed twice with PBS/0.5% bovine serum albumin before flow cytometric analysis.

Immunoblot Analysis. HEK-293, HEK-293/hOrai1/hSTIM1, Jurkat, AM1, and AM1/hOrai1 cells were harvested and rinsed twice with ice-cold 1X D-PBS, then solubilized in cell lysis buffer [1% Triton X-100, 0.1 M NaCl, 0.05 M Tris HCl (pH 8.0), 1 mM Na_2VO_4] containing Protease Inhibitor Cocktail (Roche, Branford, CT). The

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particulate material was removed by centrifugation at 14,000g for 15 minutes at 4°C, and supernatants were stored at −80°C. For detecting native conformation of hOrai1, approximately 5 μg of cell lysates in Novex Native Tris-Glycine Sample Buffer (Life Technologies) was electrophoresed in 4–20% Tris-Glycine polyacrylamide gels (Life Technologies) in Novex Tris-Glycine Native Running Buffer (Life Technologies), and transferred to nitrocellulose filters (Life Technologies). For detecting denatured hOrai1 proteins, approximately 5 μg of cell lysates in Novex Tris-Glycine SDS Sample Buffer (Life Technologies) under nonreducing or reducing conditions (NuPAGE Sample Reducing Agent; Life Technologies) was electrophoresed in 4–20% Tris-Glycine polyacrylamide gels (Life Technologies) in Novex Tris-Glycine Running Buffer (Life Technologies), and transferred to nitrocellulose filters (Life Technologies). hOrai1 proteins were detected using recombinant human anti-hOrai1 mAb 2C1.1, or polyclonal anti-Orai1 antibodies (NewEast Orai1-Li (NewEast Biosciences, Malvern, PA), NewEast Orai1-L2 (NewEast Biosciences), Alomone-Orai1-L2 (Alomone Laboratories Ltd., Jerusalem, Israel), Enzo-Orai1-L2 (Enzo Life Sciences, Plymouth Meeting, PA), or Everest-Orai1-L2 (Everest Biotech, Oxfordshire, UK). The proteins were visualized using an enhanced luminescence system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL).

**Antibody Binding Affinity Ranking Determination.** Ultra-Link Biosupport (Thermo Fisher Scientific Inc. (Pierce), Rockford, IL) was precoated with goat anti-human Fc (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), then blocked with bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Thirty picomolars anti-Orai1 antibodies were incubated with 3.0 × 10⁷, 1.0 × 10⁶, or 3.0 × 10⁵ cells/ml of AM1/hOrai1/YFP-hSTIM1 cells in 1% fetal bovine serum (FBS), 0.05% sodium azide, and Dulbecco's modified Eagle's medium (Life Technologies). Samples containing antibody (Ab) and whole cells were rocked for 4 hours at room temperature. The whole cells and antibody-cell complexes were separated from unbound free Ab using Beckman GS-6R centrifuge (Beckman Coulter, Brea, CA) at approximately 220g for 5 minutes. The supernatant was filtered through a 0.22-μm filter before being passed over the goat antihuman Fc–coated beads. The amount of bead-bound Ab was quantified by binding to fluorescent (Cy5)-labeled goat anti-human IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, Inc.) and measured using Kinetic Exclusion Assay (KinExA; Sapidyne Instruments Inc., Boise, ID). The binding signal is proportional to the concentration of free Ab in solution at each cell density. The relative binding signal of 100% represents 30 pM antibody alone. The decreased signal indicates antibody binding with AM1/hOrai1/YFP-hSTIM1 cells.

**Kinetic Exclusion Assay Affinity Measurement.** The equilibrium dissociation constant (Kd) of human anti-Orai1 mAbs were determined by the Kinetic Exclusion Assay (KinExA) method as described previously (Rathanasawami et al., 2008). Briefly, two different equilibrium sets were set up where the cells were titrated and incubated with two constant Ab concentrations, one at 20 pM and the other at 500 pM. Cell titrations and antibody solutions were set up in a final concentration of 50% whole blood. The equilibrium sets were set up where the cells were titrated and incubated for 5 minutes. The free Ab present in the supernatants of both high-Ab and low-Ab equilibrium sets were then run in triplicates over polyethyl methacrylate beads coated with goat anti-human Fc Ab, through a KinExA 3200 machine (Sapidyne Instruments Inc.). Kd was calculated from curve fitting by n-curve analysis using KinExA Pro software as reported previously (Rathanasawami et al., 2008). The 95% confidence interval is given as Kd low and Kd high.

**Aequorin-Based Calcium Assay.** Jurkat AQN cells were plated at 3 × 10⁶ cells/well (50 μl/well) in Hanks' balanced salt solution (HBSS) assay buffer (HBSS containing Ca²⁺ and Mg²⁺ + 30 mM HEPES + 30 μM coelenterazine) on CellBIND plates (Corning Incorporated) and then incubated for 2 hours at 34°C. Human anti-Orai1 mAbs were added to the wells starting at 1 μg (50 μl/well) in log dose and incubated for 1 hour at 34°C. Cells were washed three times with Ca²⁺ and Mg²⁺-free HBSS buffer and resuspended in 50 μl/well in the same buffer. Thapsigargin was added to each well (50 μl/well) at 5 μM final concentration, and the mixture was incubated at room temperature for 10 minutes. Two millimolars final concentration of calcium was then added to each well. Luminescence was measured on the FlashMIni, an in-house-developed whole-plate kinetics imaging system equipped with a multichannel dispenser.

**NFAT-Luciferase Reporter Assay.** HEK-293T/hOrai1/YFP-hSTIM1-NFAT-Luc cells were plated at 1 × 10⁵ cells/well (25 μl/well) in Dulbecco's modified Eagle's medium calcium-free medium supplemented with 10% FBS + 1X NEAA (non-essential amino acids) + 1X sodium pyruvate + 1-glutamine. Anti-Orai1 mAbs were added to the wells starting at 500 nM (25 μl/well) in log dose and incubated for 1 hour at 37°C. Thapsigargin was added to each well (25 μl/well) at 10 μM final concentration, and the mixture was incubated at 37°C for 1 hour. Two millimolars final concentration of calcium was then added to each well, and the plate was incubated for 5 hours at 37°C. Steady-Glo luciferase assay substrate (Promega Corporation) was added at 100 μl/well, and the plate was incubated at room temperature for 5 minutes and then read on a Wallac EnVision plate reader (PerkinElmer, Waltham, MA).

**Jurkat Cytokine Release Assay.** Jurkat cells were plated at 5 × 10⁵ cells/well (100 μl/well) in RPMI 1640 supplemented with 1% FBS (Life Technologies). Various concentrations of the human anti-Orai1 mAbs and isotype control mAbs were preincubated with the cells for 1 hour prior to addition of the phytohemagglutinin (PHA) or PMA + concanavalin A (ConA) stimuli. The final concentrations of PMA, PHA, and ConA (Sigma-Aldrich) were 100 nM, 2%, and 3 μg/ml, respectively. After 18 hours at 37°C, 5% CO₂, supernatant was collected, and the level of cytokine secretion was determined using a 2-spot electrochemiluminescent immunoassay from Meso Scale Discovery (MSD, Gaithersburg, MD).

To measure the amount of IL-2, 25 μl of supernatant was added to MSD plates and incubated for 1 hour. Then detection antibody was added in 130 μl of 2X Read Buffer P (Meso Scale Discovery). The plates were covered and placed on a shaking platform overnight in the dark, and electrochemiluminescence was measured using the SECTOR HTS Imager (MSD).

**Human Whole-Blood Cytokine Release Assay.** Human whole blood was obtained from healthy, nonmedicated donors in a heparin vacutainer. Various concentrations of human anti-Orai1 mAbs and isotype control mAbs were preincubated with the human whole-blood sample for 1 hour prior to addition of the thapsigargin (Alomone Laboratories) or PMA + ionomycin (Sigma-Aldrich) stimuli, resulting in a final concentration of 50% whole blood. Thapsigargin was added to a final concentration of 10 μM, and the blood was then incubated at 37°C, 5% CO₂, for 48 hours. Alternatively, blood was stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin for 24 hours. At the end of the incubation period, supernatant was collected, and the level of cytokine secretion was determined using a 4-spot electrochemiluminescent immunoassay from Meso Scale Discovery.

To determine the amount of IL-2 and IFN-γ secreted in whole blood, 25 μl of the supernatants was added to MSD Multi-Spot custom coated plates. The working electrodes on these plates were coated with capture antibodies anti-hIL-2 and anti-hIFN-γ in advance. A cocktail consisting of 20 μl of detection antibodies at 1 μg/ml each and 110 μl of 2X Read Buffer P was added. The plates were covered and placed on a shaking platform overnight in the dark, and electrochemiluminescence was measured using the SECTOR HTS Imager (MSD). To measure the amount of IL-4, IL-10, and IL-17 in whole...
blood, 25 μl of supernatant was added to MSD plates, and incubated for 1 hour. Twenty-five microliters of detection antibody diluted in antibody diluent (1 μg/ml) was added, and incubated for 1 hour. Then 110 μl of 2X Read Buffer P was added, and electrochemiluminescence was measured using the SECTOR HTS Imager (MSD). Percentage of control (POC) is a measure of the response relative to the unstimulated (the “low” value) versus stimulated (the “high” value) controls. 100 POC represents 0% inhibition of the response. In contrast, 0 POC represents 100% inhibition of the response, and would be equivalent to the response when no stimulus is given. To calculate POC, the following formula is used: [(MSD response of well) − (low)] / [(high) − (low)] × 100.

PatchXpress Recording. The currents were recorded in the whole-cell configuration using the PatchXpress 7000A automated parallel patch clamp system (Molecular Devices Inc., Sunnyvale, CA). The extracellular solution consisted of 110 mM NaCl, 10 mM CaCl₂, 3 mM KCl, 2 mM MgCl₂, 10 mM CsCl, and 10 mM HEPES (pH 7.4). The intracellular solution consisted of 95 mM cesium glutamate, 8 mM NaCl, 8 mM MgCl₂, 2 mM sodium pyruvate, 10 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid), and 10 mM HEPES (pH 7.2). All recordings were carried out at room temperature (21–23°C). The holding potential was +30 mV. The voltage protocol was as follows: 10-ms step to −100 mV followed by 100-ms ramp from −100 to +100 mV; the protocol was applied every 5 seconds. The recorded currents were leak subtracted using data collected in the extracellular solution containing 10 μM GdCl₃. The extracellular solution containing antibody was changed three times at 1-minute intervals to account for any potential binding to the cells present in the recording chamber. The percentage of I_{CRAC} current block was plotted against the antibody concentration.

Data Analysis. IC₅₀ values were calculated from a best fit of the response to a variable Hill slope using Prism software (GraphPad, San Diego, CA).

Results

Binding Characterization of Human Antihuman Orai1 Monoclonal Antibodies. U2OS cells stably overexpressing human Orai1 were used as antigen to immunize the XenoMouse (Amgen Inc., Thousand Oaks, CA) (Mendez et al., 1997). A total of 102 fully human antibodies were identified that showed specific binding to CHO-AM1 (AM1) cells stably overexpressing hOrai1 and YFP-human STIM1 (AM1/hOrai1/YFP-hSTIM1). Eleven unique recombinant monoclonal antibodies were generated and purified. All antibodies were confirmed by FACS for their specific binding to hOrai1 expressed in AM1 (AM1/hOrai1) cells. These recombinant mAbs exhibited strong specific binding to human Orai1. There was no appreciable binding to hOrai1 by the fully human antibody, DNP-3A4-F (Walker et al., 2010), which was used as an isotype control (Fig. 1A). Human anti-hOrai1 mAb 2C1.1 bound strongly to hOrai1 overexpressed on AM1 (AM1/hOrai1) cells, whereas binding to parental CHO-AM1 (AM1) cells was negligible, as shown in a representative profile in Fig. 1B.

To further validate binding specificity, we confirmed that recombinant human anti-hOrai1 mAbs also bound HEK-293 cells transiently overexpressing human Orai1 and human STIM1 (HEK-293/hOrai1+hSTIM1) (Supplemental Fig. 1). A representative binding profile is shown in Fig. 1C, indicating mAb 2C1.1 bound strongly to thapsigargin-induced HEK-293 cells coexpressing hOrai1 and hSTIM1. It should be noted that the significant staining observed relative to unstained cells in mock transfected HEK-293 cells (pcDNA3.1) stained with 2C1.1 likely indicates that the mAb recognized endogenous human Orai1 expressed on HEK-293 cells.

Fig. 1. Binding of recombinant monoclonal antibodies to human Orai1 expressed on AM1 cells. (A) FACS analysis of the parental cell line (AM1) and hOrai1-expressing cell lines (AM1/hOrai1) stained first with recombinant monoclonal antibodies then counterstained with a secondary phycoerythrin-labeled goat antihuman IgG F(ab’)2 antibody. (B) Binding profiles of AM1 and AM1/hOrai1 cells, unstained, stained with mAb 2C1.1 and isotype control antibody, DNP-3A4-F. (C) Binding profiles of mAb 2C1.1 to thapsigargin-induced HEK-293 cells transiently coexpressing hOrai1 and hSTIM1 or with pcDNA3.1 vector transfected HEK-293 cells. FL2-H, the height of the pulse detected by a 585/42 band pass filter used with the 488 nm laser; RFI, relative fluorescence intensity.
To extend our understanding of binding to native Orai1, we examined the hOrai1 mAbs for their ability to detect human Orai1 on the surface of Jurkat and HEK-293 cells, as endogenous \( I_{\text{CRAC}} \) is known to be present in these cells (Fasolato and Nilius, 1998; Sternfeld et al., 2007). All of the tested antibodies recognized endogenously expressed Orai1 on the surface of Jurkat and HEK-293 cells (unpublished data). Representative results are shown in Fig. 2 (A and B) demonstrating the binding of mAb 2C1.1 to hOrai1 on Jurkat and HEK-293 cells, but not the isotype control antibody, anti-KLH mAb (Walker et al., 2010). In addition, we confirmed that mAb 2C1.1 detected human Orai1 on human CD3+ T cells (Supplemental Fig. 2).

Several commercially available polyclonal anti-Orai1 antibodies to human Orai1 raised against various peptides from the hOrai1 ECL1 and ECL2 domains were also assessed for binding by FACS (NewEast Orai1-L1, NewEast Orai1-L2, Alomone-Orai1-L2, Enzo-Orai1-L2, and Everest-Orai1-L2) to human Orai1 on AM1/hOrai1/YFP-hSTIM1 cells. No detectable binding to human Orai1 was observed with any of the commercially available polyclonal antibodies (unpublished data).

Orai1 has two human paralogs, Orai2 and Orai3 (Gwack et al., 2007). The conservation of the two extracellular loops between Orai1 and the two human paralogs is less than 70%. We characterized the human anti-hOrai1 mAbs for selectivity in binding to Orai1 versus Orai2 and Orai3. Human anti-hOrai1 mAb 2C1.1 exhibited strong binding to hOrai1, but failed to recognize hOrai2 and hOrai3 expressed in HEK-293 cells transiently, as shown in a representative profile in Fig. 3A. Subsequently, our fully human antibodies were evaluated for binding to rodent Orai1. The recombinant mAbs specifically bound human Orai1 and did not recognize mouse or rat Orai1 (Fig. 3B).

Human Orai1 has two common SNPs in the extracellular loop 2 domain. One variant has an SNP encoding a serine-to-glycine substitution at position 218 of hOrai1 (S218G, NCBI SNP database rs3741596), and the other is an SNP encoding an asparagine-to-serine substitution at position 223 of hOrai1 (N223S, NCBI SNP database rs75603737). The frequency of heterozygosity for S218G and N223S calculated from 306 individuals representing different ethnic groups is about 20% and 6%, respectively. Interestingly, chimpanzee Orai1 has a glycine at position 218. Our recombinant mAbs were assessed for their ability to distinguish among the different SNP variants of the human Orai1 protein. There was no discernable difference in binding by the fully human mAbs to wild-type human Orai1 or either of the common SNP variants, as shown in Fig. 3C, by mAb 2C1.1. The amino acid sequence of human and cynomolgus Orai1 is identical in the two extracellular loops, and the recombinant anti-human Orai1 mAbs also recognized Orai1 on cynomolgus T cells (Gaida K et al., manuscript in preparation) as well as cynomolgus Orai1 expressed in HEK-293 cells transiently (unpublished data).

To further assess recombinant human anti-hOrai1 mAbs for their ability to detect native hOrai1, HEK-293, HEK-293/hOrai1/hSTIM1, Jurkat, AM1/CHO, and AM1/hOrai1 cell lysates were separated by native gel electrophoresis and examined by Western blot using mAb 2C1.1. A high-molecular-weight immunoreactive species corresponding to oligomers of hOrai1 was detected in HEK-293/hOrai1/hSTIM1 and AM1/hOrai1 cell lysates (Fig. 4A). These results illustrate that the mAb detects hOrai1 in its native conformation, and these data are consistent with our FACS binding results.

Furthermore, the recombinant anti-hOrai1 antibodies were examined by SDS-PAGE for their ability to identify denatured hOrai1 under reducing and nonreducing conditions. Western blots using mAb 2C1.1 revealed an immunoreactive species of approximately 48 kDa corresponding to the expected size for the glycosylated form of hOrai1 in HEK-293/hOrai1/hSTIM1 and AM1/hOrai1 cell lysates (Fig. 4B). The mAb 2C1.1 also detected two lighter bands at approximately 33 and 96 kDa, corresponding to the molecular weight of the predicted unglycosylated hOrai1 and oligomeric hOrai1, respectively (Fig. 4B). All of the commercially available antibodies we tested were unable to detect hOrai1 proteins under native conditions (unpublished data), but the NewEast Orai1-L1 and NewEast Orai1-L2 antibodies detected an immunoreactive species of approximately 48 kDa (Fig. 4, C and D) when run under denaturing conditions (reduced or nonreduced) corresponding to the expected size for the glycosylated form of hOrai1. In contrast, no specific immunoreactive species were detected by the Alomone-Orai1-L2, Enzo-Orai1-L2, and Everest-Orai1-L2 antibodies (unpublished data). Since hamster AM1 cells do not express endogenous Orai1, the 33-kDa band detected in HEK-293, HEK-293/hOrai1/hSTIM1, Jurkat, AM1, and AM1/hOrai1 cells with all the commercial antibodies likely represents a nonspecific crossreactive protein (Fig. 4, C and D). As this band has often been assumed to correspond to hOrai1 in
Various publications, these observations highlight that findings with commercially available antibodies should be interpreted with caution unless the antibodies have been robustly characterized for specificity.

Characterization of Binding Affinity. KinExA assays were run to determine the rank order of affinity of the recombinant anti-hOrai1 mAbs for binding to the native human Orai1 expressed on a stable AM1/hOrai1/YFP-hSTIM1 cell line. Figure 5 shows the percentage of free mAb was 100% when no cells were added, whereas the percentage of free mAb progressively decreased with increasing density of added AM1/hOrai1/YFP-hSTIM1 cells, indicating that the anti-hOrai1 mAbs bound to hOrai1 on the cells. In each set, as expected, the higher-affinity binders showed a lower percentage of free mAb when compared with the low-affinity binders. Based on this analysis, the rank order of binding affinity for the human anti-hOrai1 mAbs was 5F7.1 > 5H3.1 > 2C1.1 = 2B7.1 = 5A4.2 > 5B1.1 = 5B5.1 = 5D7.2 > 5F2.1 > 2D2.1 > 2B4.1. Four representative human anti-hOrai1 mAbs, 2C1.1, 2D2.1, 2B7.1, and 5F7.1, were selected to determine individual Kd values by KinExA. The Kd values of 2C1.1, 2D2.1, 2B7.1, and 5F7.1 for binding to native human Orai1 expressed on the stable mammalian cell line AM1/hOrai1/YFP-hSTIM1 were 40, 99, 100, and 19 pM, respectively (Table 1). The mAb 2B4.1 was a weak binder with an affinity of approximately 1.8 nM by FACS Kd affinity measurement (unpublished data).

Epitope Mapping. Human, rat, and mouse Orai1 proteins differ by only two residues in the extracellular loop 1 region, but exhibit less than 60% amino acid identity in the extracellular loop 2 region. Since our anti-hOrai1 mAbs do not recognize mouse or rat Orai1 (Fig. 3B), we speculated that binding may occur within ECL2, which differs the most between human and rodent Orai1. To test this hypothesis, we generated two chimeras (Ch.), hOrai1-mOrai1 ECL2 and mOrai1-hourOrai1 ECL2, and tested the binding of four representative mAbs, 2C1.1, 2D2.1, 2B7.1, and 5F7.1, to these chimeric proteins.

The hOrai1-mOrai1 ECL2 chimera is an ortholog hybrid where only the ECL2 region of the human Orai1 is replaced with the mouse Orai1 ECL2 sequence. The mOrai1-hourOrai1 ECL2 chimera is the opposite configuration where only the ECL2 region of mouse Orai1 is replaced with the human ECL2 region. Table 2 shows that the recombinant anti-hOrai1 mAbs that failed to bind to full-length mouse Orai1 bound strongly to the mOrai1-hourOrai1 ECL2 chimera (Ch. 12), indicating that the epitope lies exclusively in the human Orai1 ECL2 region. Supporting this conclusion, the mAbs exhibited no binding to the hOrai1-mOrai1 ECL2 chimera (Ch. 1) where the ECL2 region of human Orai1 is replaced with mouse residues (Table 2).

To more precisely determine the region within the human extracellular loop 2 domain that the anti-hOrai1 monoclonal antibodies bind to, we generated a series of additional mutants (Tables 2 and 3) and transiently expressed them in HEK-293 cells to map the binding epitope. We designated “gain-of-binding” hOrai1-mOrai1 ECL2 chimeras as Ch. 2 to Ch. 11 in Table 2, where selected amino acids in the mouse Orai1 ECL2 region were replaced with human residues in an attempt to restore binding. Table 2 shows that none of the recombinant mAbs bound to chimeras Ch. 7 through Ch. 11. Earlier we demonstrated that the fully human mAbs bound...
equally well to two SNP variants. This suggests that the region from residues 218 to 226 is unlikely to play a major role in the binding of our mAbs to Orai1. There was a partial, but substantial, gain of binding observed with the Ch. 6 chimera (KPPAE \to SKPPA) by mAbs 2B7.1, 2C1.1, and 2D2.1, indicating that this region from amino acid residues 213 to 217 of hOrai1 ECL2 was important for their binding. Interestingly, the gain of binding to Ch. 6 by mAb 5F7.1 was comparable to the observed full binding to Ch. 12 (Table 2) and human Orai1. This indicates significant differences in specificity between recombinant mAbs 2B7.1, 2C1.1, and 2D2.1, and mAb 5F7.1.

As the humanization of the mouse ECL2 region was expanded, all four recombinant mAbs bound strongly; binding to Ch. 5 containing amino acid changes in the 210–217 region was comparable to binding to Ch. 12. Further extension of these changes in the amino- or carboxy-terminal direction (Ch. 2 to Ch. 4) did not further enhance binding over observed binding to Ch. 5 (Table 2). Taken together, the gain-of-binding data suggest that amino acid residues 210–217 of hOrai1 ECL2 are critical for binding by our fully human anti-hOrai1 monoclonal antibodies.

To further confirm and extend the results seen in our gain-of-binding experiments, we generated a series of “loss-of-binding” mOrai1-hourOrai1 ECL2 chimeras (Ch. 13 to Ch. 21; Table 3) in which specific amino acid residues of the hOrai1 ECL2 region were replaced with mouse Orai1 residues. Earlier we demonstrated that the human ECL2 region was sufficient for binding by all the mAbs since they bound fully to the mOrai1-hourOrai1 Ch.12 molecule which was humanized over the entire ECL2 region, but did not recognize native mouse Orai1 (Ch. 1; Fig. 3B; Table 3). Substitution of human Orai1 amino acid sequence between residues 204–217 of ECL2 (Ch. 13 to Ch. 17) with their mouse counterparts caused complete loss of mAb binding (Table 3), verifying that this portion of the hOrai1 ECL2 region is important for the binding to Ch. 5 (Table 2). Taken together, the gain-of-binding data suggest that amino acid residues 210–217 of hOrai1 ECL2 are critical for binding by our fully human anti-hOrai1 monoclonal antibodies.

To further confirm and extend the results seen in our gain-of-binding experiments, we generated a series of “loss-of-binding” mOrai1-hourOrai1 ECL2 chimeras (Ch. 13 to Ch. 21; Table 3) in which specific amino acid residues of the hOrai1 ECL2 region were replaced with mouse Orai1 residues. Earlier we demonstrated that the human ECL2 region was sufficient for binding by all the mAbs since they bound fully to the mOrai1-hourOrai1 Ch.12 molecule which was humanized over the entire ECL2 region, but did not recognize native mouse Orai1 (Ch. 1; Fig. 3B; Table 3). Substitution of human Orai1 amino acid sequence between residues 204–217 of ECL2 (Ch. 13 to Ch. 17) with their mouse counterparts caused complete loss of mAb binding (Table 3), verifying that this portion of the hOrai1 ECL2 region is important for the binding to Ch. 5 (Table 2). Taken together, the gain-of-binding data suggest that amino acid residues 210–217 of hOrai1 ECL2 are critical for binding by our fully human anti-hOrai1 monoclonal antibodies.
binding by our recombinant human anti-hOrai1 mAbs. In support of the gain-in-binding studies (Table 2), the loss-of-binding mutants confirmed that a narrow region between residues 210–217 of hOrai1 was essential for antibody recognition (Ch. 14 and Ch. 15; Table 3). Consistent with our earlier findings, amino acid residues 219–226 of hOrai1 were unimportant for binding by our mAbs, as the mouse chimeric changes in this region (Ch. 20 and Ch. 21) showed no effect on binding.

There were some subtle differences among the antibody clones; recombinant mAbs 2B7.1, 2C1.1, and 2D2.1 exhibited a slightly different binding profile to chimeras Ch. 18 and Ch. 19 than mAb 5F7.1 (Table 3). Whereas mAbs 2B7.1, 2C1.1, and 2D2.1 showed some weak binding to Ch. 18 and Ch. 19 mutants (Table 3), mAb 5F7.1 failed to bind to either of these chimeras (Table 3). This suggests that amino acid residues 216 and 217 of hOrai1 ECL2 play a minor role in binding by mAbs 2B7.1, 2C1.1, and 2D2.1, whereas these residues are critical for mAb 5F7.1 binding.

In summary, the gain-of-binding experiments were consistent with the loss-of-binding experiments, and defined the epitope for our monoclonal antibodies to a narrow sequence of amino acids, 210–217, of the hOrai1 ECL2 region. Although the gross footprint for binding by these four mAbs was the same, the emphasis of the binding was slightly different in that mAb 5F7.1 bound more strongly to a subregion from residues 213–217 (Tables 2 and 3). In contrast, the binding to human Orai1 by mAbs 2B7.1, 2C1.1, and 2D2.1 emphasized a slightly different subset of residues, shifted more to the left to include residues 210–215 of the hOrai1 sequence.

**Functional Characterization of Human Anti-hOrai1 mAbs.** To characterize the functional impact of our fully human anti-hOrai1 mAbs, the antibodies were tested for their effect on $I_{\text{CRAC}}$ current and calcium influx, in addition to examining their impact on T cell NFAT activation and cytokine production. Because Orai1 currents are small and electrophysiology is a low-throughput assay, the anti-hOrai1 mAbs were first tested for impact on store-operated calcium flux and T cell cytokine responses, and only those showing inhibitory activity were further characterized for impact on $I_{\text{CRAC}}$ current.

Calcium influx through CRAC leads to sustained increases in intracellular calcium that are essential for NFAT activation and T cell cytokine production, but the CRAC channel can also be inactivated by elevated intracellular calcium. Mitochondria, however, located near cell surface CRAC channels, can help prevent channel inactivation by rapid uptake of calcium, thereby allowing sustained store-operated Ca$^{2+}$ entry after antigen stimulation of T lymphocytes (Hoth...
et al., 2000; Parekh, 2008). Using Jurkat cells stably expressing aequorin localized to the mitochondria (JurkatAQN), we examined the effect of the fully human anti-hOrai1 monoclonal antibody 2C1.1 on store-operated Ca\textsuperscript{2+} entry. As shown in Fig. 6A, mAb 2C1.1 reduced Ca\textsuperscript{2+} influx in a dose-dependent fashion by approximately 70% in JurkatAQN cells with an IC\textsubscript{50} of 1.16 nM, whereas the nonfunctional mAb, 2B4.1, and isotype control mAb, DNP-3A4-F, demonstrated no inhibitory effect.

Sustained calcium influx via CRAC channels leads to a signaling cascade that results in the activation of several transcription factors, with perhaps the best characterized being NFAT. We assessed the ability of the human Orai1 mAb 2C1.1 to block NFAT activity using an NFAT-luciferase reporter assay. We generated a recombinant stable cell line which expresses human Orai1 and human STIM1 along with an NFAT-driven luciferase reporter (HEK-293T/hOrai1/YFP-hSTIM1-NFAT-Luc). As shown in Fig. 6B, mAb 2C1.1 dose dependently reduced NFAT-luciferase activity by approximately 50% in response to thapsigargin-stimulated HEK-293T/hOrai1/YFP-hSTIM1-NFAT-Luc cells, with an IC\textsubscript{50} of 4.5 nM, whereas the isotype control mAb, DNP-3A4-F, did not demonstrate an inhibitory effect.

The human T cell leukemia line, Jurkat, has been routinely used as a model for studying T cell activation in vitro. We evaluated whether mAb 2C1.1 inhibited IL-2 production from either PHA or PMA + ConA–stimulated Jurkat cells. Recombinant mAb 2C1.1 dose dependently suppressed IL-2 secretion, with IC\textsubscript{50} values of 0.28 and 1.7 nM from Jurkat cells stimulated with PHA and PMA + ConA, respectively. The levels of suppression were 70% and 60%, respectively, from these two stimuli. No inhibitory effect was detected by the isotype control mAb, DNP-3A4-F (Fig. 7, A and B).

The human Orai1–binding antibodies were also tested for their ability to inhibit T cell activation in human whole blood in vitro, as described earlier (Sullivan et al., 2010). In brief, 50% human whole blood is stimulated with thapsigargin to induce store depletion, calcium mobilization, and cytokine secretion. The anti-Orai1 monoclonal antibodies were pre-incubated with the human whole blood for 30–60 minutes before stimulation.

### Table 2

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Table 2

Binding of human anti-hOrai1 mAbs to hOrai1-mOrai1 ECL2 chimera gain-of-binding mutants as determined by FACS

An alignment between the human and mouse Orai1 protein in the ECL2 region only with the human amino acids represented in capital letters. The mouse ECL2 amino acids in lowercase letters and the underlined amino acids denote differences between human and mouse protein sequences. The human-to-mouse amino acid changes in the table are represented by lowercase letters, and the dashes denote no changes. ++++ denotes comparable binding of antibodies to chimeras and human Orai1. ** denotes antibodies show weak binding to chimeras as compared to human Orai1.

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Table 3

Binding of monoclonal antibodies to mOrai1-hOrai1 ECL2 chimera loss-of-binding mutants as determined by FACS

An alignment between the human and mouse Orai1 protein in the ECL2 region only with the human amino acids represented in capital letters. The mouse ECL2 amino acids in lowercase letters and the underlined amino acids denote differences between human and mouse protein sequences. The human-to-mouse amino acid changes in the table are represented by lowercase letters, and the dashes denote no changes. ++++ denotes comparable binding of antibodies to chimeras and human Orai1. ** denotes antibodies show weak binding to chimeras as compared to human Orai1.
prior to TG stimulus, and the level of cytokine secretion was determined 48 hours later using an electrochemiluminescent immunoassay. With the exception of the low-affinity binder mAb 2B4.1, the anti-Orai1 mAbs caused a dose-dependent suppression of IL-2 and IFN-\(\gamma\) release from human blood, resulting in >80% inhibition of the response. The half-maximal inhibitory concentrations (IC\(_{50}\)) of human Orai1 functional mAbs were in the picomolar to low nanomolar range (Table 4; \(n = 6–9\) donors). Figure 8 shows representative dose-inhibition curves for IL-2 and IFN-\(\gamma\) secretion for the mAbs 2D2.1, 2C1.1, and 2B7.1. These mAbs provided nearly full blockade of cytokine release. In contrast, the low-affinity anti-hOrai1 mAb 2B4.1, and the isotype control, DNP-3A4-F, had no inhibitory effect on either IL-2 or IFN-\(\gamma\) release (Fig. 8).

To further validate the function of recombinant mAb 2C1.1 in inhibiting human T cell activation in 50% human whole blood, we tested its effect on cytokine production from three different donors stimulated with PMA + ionomycin. This stimulus induced a robust IL-4, IL-10, and IL-17 cytokine response that was almost fully blocked by mAb 2C1.1. The concentration of mAb 2C1.1 needed to inhibit 50% of the cytokine response (IC\(_{50}\)) was 1.66 ± 1.41 nM, 1.18 ± 1.35 nM and 2.99 ± 1.29 nM, respectively, for IL-4, IL-10, and IL-17 (Fig. 9, A–C; \(n = 3\) donors). The anti-KLH isotype control showed no impact on the cytokine responses.

To demonstrate that the antibodies were mediating their effect through blockade of channel, mAb 2C1.1 and a second functionally active antibody, 2D2.1, were evaluated by electrophysiology for their direct inhibition on \(I_{\text{CRAC}}\) currents using HEK-293 stably coexpressing hOrai1 and hSTIM1 (HEK293/hOrai1/hSTIM1). All experiments were carried out with the PatchXpress 7000A planar patch-clamp system from Molecular Devices Corporation. Upon store depletion with BAPTA, large-membrane currents (Fig. 10B) with inwardly rectifying current-voltage (I-V) relationships (Fig. 10A) were observed that are characteristic of \(I_{\text{CRAC}}\). In initial studies, the CRAC currents were partially blocked by a single
concentration (1 μM) of mAb 2C1.1 (39% ± 4.9% inhibition; n = 7; Fig. 10, A and B) and mAb 2D2.1 (50.4% ± 6.5% inhibition; n = 9; unpublished data). To extend these findings, the inhibitory effect of mAb 2C1.1 antibody on I_{CRAC} current was measured at six concentrations of mAb 2C1.1 (n = 3–6 each). The percentage of I_{CRAC} current block was plotted against the antibody concentration. The IC_{50} was calculated as 56.3 ± 1.1 nM (Fig. 10C). In contrast, the isotype control antibody (DNP-3A4-F) applied at 1 μM concentration did not show a significant effect on the I_{CRAC} current (Fig. 10A).

**Discussion**

For human therapeutic drug development, ion channels have traditionally been targeted using small-molecule approaches, and the CRAC channel is no exception. Although several small molecules have been shown to be potent CRAC channel inhibitors (Ishikawa et al., 2003; Zitt et al., 2004; Yonetoku et al., 2006a,b, 2008; Yoshino et al., 2007), the full selectivity of these compounds for CRAC versus other ion channels has not been fully described, nor have any of them reached the stage of human clinical trials. In this study, we demonstrated a successful immunologic approach to CRAC channel inhibition through the development of fully human monoclonal antibodies to hOrai1 that antagonize CRAC channel function.

We examined the selectivity of recombinant anti-hOrai1 mAbs for binding to human paralogs and rodent orthologs, and determined the binding epitope within human Orai1. The 11 unique recombinant mAbs were selective for human Orai1, and did not recognize human Orai2 or Orai3 (Fig. 3A), nor the mouse or rat Orai1 orthologs (Fig. 3B). In addition, we validated by FACS that these recombinant mAbs bind wild-type hOrai1 (Fig. 1), and are not impacted by two common SNP variants (Fig. 3C). Moreover, we confirmed that our recombinant mAbs bind to endogenous hOrai1 on HEK-293, Jurkat (Fig. 2), and primary human CD3^{+} T cells (Supplemental Fig. 2), and by Western blot, we demonstrated that the anti-hOrai1 mAbs recognize both native and denatured hOrai1 protein (Fig. 4).

KinExA affinity measurements indicate that the fully human anti-hOrai1 mAbs were high-affinity binders, with K_{d} values in the 20–100 μM range (Table 1), with the exception of mAb 2B4.1 (Fig. 5). The lack of binding of the fully human anti-hOrai1 mAbs to mouse or rat Orai1 suggested that the mAbs bind to the ECL2 of hOrai1, which is poorly conserved at the amino acid sequence level across species (Fig. 3). A detailed epitope mapping strategy using point mutations in the ECL2 region of human and mouse Orai1 confirmed this hypothesis, and revealed that amino acid residues 210–217 of the ECL2 region of human Orai1 are critical for binding by our recombinant human anti-hOrai1 mAbs. These results as a whole validate the concept of antibodies as a modality for selective targeting of ion channels.

The recent crystal structure of Orai from *Drosophila melanogaster* reveals that the CRAC channel is composed of a hexameric assembly of Orai subunits with transmembrane (TM) segment 1 forming an inner ring that contributes to the ion-conduction pore. The TM2 and TM3 domains constitute a middle ring that separates TM1 from TM4, TM4 forming the outer ring of the channel (Hou et al., 2012). STIM is proposed to interact with TM1 and TM4 of Orai to activate the channel through a proposed conformational change in TM4. Our recombinant mAbs showed inhibitory activity on open CRAC channels in electrophysiology experiments. This suggests that binding by the antibody may play a role in the conformational change of TM4, thus restoring a closed state to the channel. How this would be specifically accomplished will require

**TABLE 4**

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<th>IFN-γ IC_{50} (nM)</th>
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<td>0.86 ± 0.62</td>
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<td>2B7.1</td>
<td>0.90 ± 0.59</td>
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Data represent the mean ± S.D., calculated from 6–9 different donors.
further biophysical studies with the antibody and specific channel mutants.

Eleven function-blocking polyclonal antibodies have been previously reported from nine different ion channels, all targeting the E3 extracellular loop of the channel (Naylor and Beech, 2009). The reported antibodies were generated to 20-mer peptides of the turret region of the ion channels in question. In most cases, the E3 antibodies produced only
a partial blockade of channel function. For polyclonal antibodies, the binding epitope is unlikely to be located in a specific region of the E3 extracellular loop, and the affinity of the reported E3 antibodies is not fully described.

Most of our recombinant mAbs also exhibited potent, but partial, inhibition of CRAC channel function (Figs. 6, 7, and 10). We show that our functional mAbs are high-affinity binders and bind to a specific region of hOrai1 ECL2. The knowledge gained from affinity measurements and epitope mapping has contributed to our understanding of the requirements for antagonist activity against the CRAC channel.

The mAb 2B4.1, which did not block T cell function, was a weak binder, with a Kd of approximately 1.8 nM by FACS affinity measurement (unpublished data), and was more than 10-fold weaker in binding compared with functional blockers in KinExA affinity measurement (Fig. 5). By epitope mapping, mAb 2B4.1 binds to a specific epitope in extracellular loop 2, similar to the functional blockers (unpublished data). However, mAb 2B4.1 did not inhibit interleukin-2 or interferon-γ secretion in thapsigargin-treated human whole blood (Fig. 8), and was inactive in blocking store-operated Ca2+ entry in JurkatAQN cells (Fig. 6A). Based on these results, we propose that, for antibodies to inhibit CRAC, they need to exhibit high-affinity binding and recognize a specific epitope within the extracellular loop 2 region of hOrai1.

Our recombinant human anti-hOrai1 mAbs robustly inhibited cytokine secretion by human T cells in whole blood after thapsigargin or PMA + ionomycin stimulation, showing potency values in the picomolar to low nanomolar range (Figs. 8 and 9; Table 4). Depletion of ER Ca2+ stores activates CRAC channels, resulting in Ca2+ influx and activation of NFAT, which subsequently instigates transcription of various cytokines (Okamura et al., 2000; Feske et al., 2003). We demonstrated that human anti-hOrai1 mAbs dose dependently reduced ICRAC currents (Fig. 10), attenuated store-operated Ca2+ influx (Fig. 6A), and suppressed NFAT-luciferase activity (Fig. 6B), albeit partially. We further validated the functional activity of recombinant mAb 2C1.1 in inhibiting cytokine production from Jurkat T cells stimulated with PHA or PMA + ConA. Recombinant mAb 2C1.1 dose dependently suppressed IL-2 production (Fig. 7), with IC50 values comparable to those observed in the whole-blood cytokine assays. Our experiments demonstrate that our recombinant human anti-hOrai1 mAbs are functional CRAC channel blockers, albeit partial in some assays. The partial inhibition in some in vitro assays was sufficient for a nearly full block of cytokine production in human whole blood following thapsigargin or PMA + ionomycin stimulation. The biology behind this difference is not fully understood.

The ability of mAb 2C1.1 to block cytokine production in human PBMCs (peripheral blood mononuclear cells) stimulated with αCD3 or αCD3/αCD28 was unfortunately inconclusive, as several isotype control antibodies also exhibited inhibitory effects in those assays.

The observed blockade of the anti-inflammatory cytokine, IL-10, in addition to proinflammatory cytokines raises the possibility that inhibition of the CRAC channel with mAb 2C1.1 will not lead to full inhibition of an integrated immune response in vivo. However, it should be noted that the clinically validated immunosuppressive drug, cyclosporine, has also been shown to inhibit IL-4, IL-10, and IL-17 secretion in these same assays (unpublished data).

In summary, we successfully explored a novel approach targeting the CRAC channel, and developed 10 human anti-hOrai1 mAbs, represented by recombinant mAb 2C1.1, that have high affinity and are human Orai1–selective binders which can antagonize CRAC channel function. More importantly, the inhibitory effect of our recombinant human anti-hOrai1 mAbs has been demonstrated not only on Jurkat T cells but also in human whole blood following either thapsigargin induction or PMA plus ionomycin stimulation. These results are consistent with our hypothesis that anti-CRAC antibodies have potential as therapeutics for T cell–driven autoimmune disease, and this hypothesis can be further tested in an in vivo setting.

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Authorship Contributions


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Supplemental Data

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Generation and Characterization of Fully Human Monoclonal Antibodies Against Human Orai1 for Autoimmune Disease

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Supplemental Figure 1. Binding of recombinant monoclonal antibodies to thapsigargin induced HEK-293 cells transient co-expressing hOrai1 and hSTIM1 along with pcDNA3.1 vector transfected HEK-293 cells.

Supplemental Figure 2. Recombinant human anti-hOrai1 recognizes endogenous hOrai1 on human T cells. Binding profiles of CD3+ T cells, unstained, stained with Alexa Fluor 647 labeled mAb 2C1.1 or Alexa Fluor 647 labeled isotype control antibody, anti-KLH.
Supplemental Figure 1

A.

[Graph showing data points for various mAbs, 10 μg/ml, with labels for HEK-293/pcDNA3.1 and HEK-293/hOral1+hSTIM1.]
Supplemental Figure 2

A.

Count

Alexa Fluor 647-A

-10^2 -10^1 10^2 10^3 10^4 10^5

CD3+ T cells, unstained
CD3+ T cells + AF647-2C1.1
CD3+ T cells + AF647-anti-KLH