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Production of a monoclonal antibody against the alpha 4 beta 7 integrin

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PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST THE ALPHA 4 BETA 7 INTEGRIN

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PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST THE ALPHA 4 BETA 7 INTEGRIN

by
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ABSTRACT

PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST THE ALPHA 4 BETA 7 INTEGRIN

by Asima Khan

This study describes an attempt to produce a monoclonal antibody (mAb) against the human alpha 4 beta 7 ($\alpha4\beta7$) gut-homing integrin found on lymphocytes. The recruitment of lymphocytes via the $\alpha4\beta7$ receptor to the gut has been associated with inflammatory bowel disease (IBD). A novel immunization strategy was employed in generating polyclonal antibodies (pAb) in C57BL/6 mice. $\beta7$ knock-out (K/O) mice were immunized with $\beta7$ wild-type (WT) mouse splenocytes with cell-surface $\beta7$. As a final boost, mice were injected with human peripheral blood mononuclear cells (PBMC), with a rationale of boosting epitopes shared between murine and human $\beta7$. Immune sera and clones generated by hybridoma technology were screened using the enzyme-linked immunosorbent assay (ELISA) and flow cytometry. Despite binding to both mouse cells and PBMCs expressing $\alpha4\beta7$, the mAb produced is unlikely to be anti-$\alpha4\beta7$ because it generated a different staining pattern than the positive control, ACT-1, on T cell subsets that migrate to the gut.

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I will forever be grateful for my parents who unconditionally love and believe in me and for their tremendous help in times of need. I'd also like to thank my dear husband, Raafay, for his encouragement, support and motivation throughout these three years. Above all, I'd like to thank God for making this possible for me. He makes the difficult easy.

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PREFACE

The following thesis consists of three chapters and the appendices. The first chapter is a detailed introduction of inflammatory bowel disease, the mechanism of lymphocyte homing, the role of the alpha 4 beta 7 integrin, strategies for treating the disease and our research goals. The second chapter is presented in manuscript format in accordance with guidelines from the Journal of Immunology. Chapter II has its own separate list of references cited. Chapter III is a detailed discussion of the results and future direction for the study. Chapter III is followed by a list of references cited for both Chapters I and III. The appendices provide supplementary materials associated with the research.

CHAPTER I

INTRODUCTION

INTRODUCTION

Inflammatory Bowel Disease

IBD is comprised of Crohn's disease (CD) and ulcerative colitis (UC), two chronic intestinal inflammatory conditions. UC involves inflammation of the inner lining of the large intestine or colon while CD extends deeper into the intestinal wall and commonly affects both the large and small intestine and other organs in the digestive tract (1). Patients typically experience lifelong recurring symptoms such as bloody diarrhea and abdominal cramping during bowel movements. There are an average of 6 new cases of CD and 12 new cases of UC per 100,000 people per year (2, 3).

Although the etiology of IBD is still elusive, a genetic predisposition, environmental factors and an aberrant immune response are all implicated in influencing IBD development (4). Studies of IBD in recent years have led to a better understanding of its pathogenesis and various aspects of the regulation of the mucosal immune system. IBD is manifested by chronic mucosal inflammation and an influx of inflammatory leukocytes. Basically, a heightened mucosal immune reaction occurs in response to harmless bacterial antigens (5). A variety of immunologic defects, such as an excessive T and B cell response, have been identified.

In healthy individuals, the immune system is able to distinguish between commensal bacteria that maintain a symbiotic relationship with their hosts and pathogens (6). Receptors expressed by dendritic cells (DC), toll-like receptors (TLR), recognize structurally conserved molecular patterns found on microbes. Using TLRs, DCs are able to

polarize naïve T cell differentiation into regulatory T cells (in response to the commensal bacteria) or into effector T cells (in response to pathogens) (4).

In IBD, several abnormalities of the mucosal immune system have been cited (7). First of all, the expression of TLRs goes awry. TLRs that react in the presence of pathogens such as TLR4 are up-regulated, while those that maintain homeostasis such as TLR3 are down-regulated and suppressed (4). Also, DCs falsely respond to indigenous microbial antigens and undergo differentiation leading to disproportionate populations of effector T cells and pro-inflammatory responses (8). Pro-inflammatory cytokines such as IL-12, IL-18, IL-23, tumor necrosis factor alpha (TNF-α) and IFN γ are released and up-regulate the expression of adhesion molecule ligands on the vascular endothelium encouraging leukocyte adhesion and extravasation into the tissue (9-11). T and B lymphocytes and macrophages are among the leukocytes that respond to these chemotactic signals. Upon reaching the antigen site, macrophages then release chemokines that accumulate at endothelial cell surfaces and attract more inflammatory leukocytes, continuing the cycle. An interplay of all these immunologic events leads to perpetual inflammation in what is known as IBD (5).

Lymphocyte Homing

The recruitment process of lymphocytes to the gut involves several sequential steps (Fig. 1) (12). The cells are in transit, under high flow conditions in the bloodstream and must come to an arrest and proceed through the vasculature to the tissue of interest (13). The first step is the primary adhesion which is transient and reversible, accomplished by selectins found on lymphocytes binding their ligands on the endothelial cell surface (14).

This interaction allows for loose rolling of the lymphocytes along the vessel wall and successfully slows their transit. Shortly after the initial contact, the lymphocytes are activated by chemokines on the endothelial surface interacting with chemokine receptors found on the cells (15, 16). This interaction increases the affinity of integrin receptors for their adhesion molecules and promotes their binding. Specifically, the $\alpha 4\beta 7$ integrin receptor on the lymphocyte cell-surface binds the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) found on the endothelium. This results in an activation-dependent arrest and firmly adheres the lymphocytes to the endothelium. Finally, cells undergo transmigration, the passage of lymphocytes through intact vessel walls to the surrounding tissue (17-19).

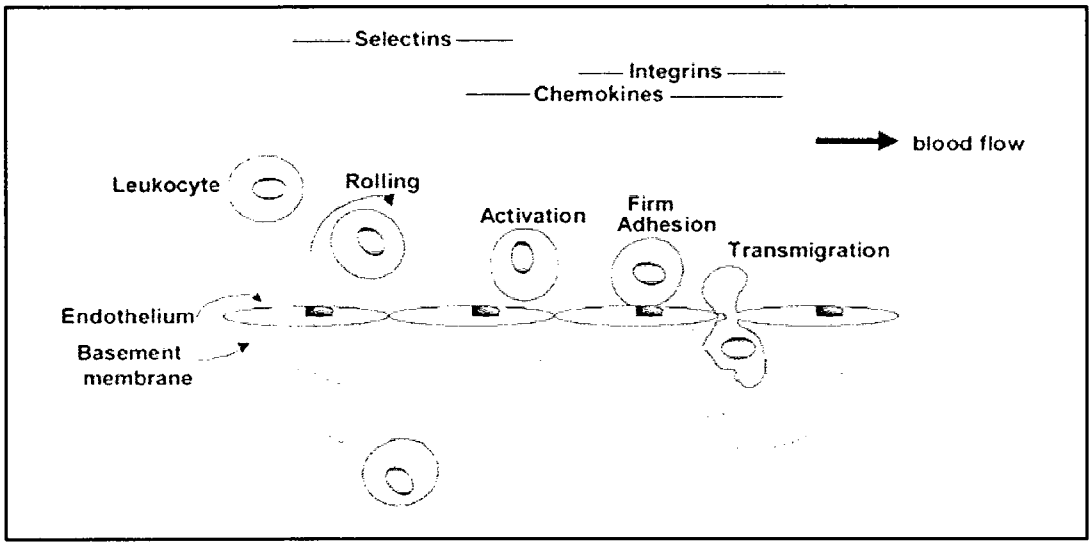

FIGURE 1. A multi-step model of leukocyte extravasation. This figure was reprinted with permission from Dr. Patricia Celie, Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, the Netherlands. Endothelial cells express selectin ligands triggered by inflammation. Selectins are expressed on leukocytes, such as L-selectin (not shown), and transiently interact with their ligands resulting in a rolling motion over the endothelium. This effectively slows the cells down which are in transit in the bloodstream. Then, chemokines generated at the endothelial surface, such as CCL25 (not shown), bind with chemokine receptors found on the leukocytes, such as CCR9 (not shown), resulting in leukocyte activation. Shortly afterwards, the leukocyte integrins, such as $\alpha 4\beta 7$ (not shown), are activated by binding to their ligands, such as MAdCAM-1(not shown), on the endothelium. This results in firm adhesion of the cell to the endothelial surface. Transmigration of the cell across the endothelial barrier follows.

Integrins and α4β7

The integrins, a family of cell adhesion receptors, are comprised of two non-covalently linked transmembrane glycoprotein subunits, α and β, that form heterodimers (20). Intergin receptors mediate interactions of the cells with endothelial tissue via ligands called intercellular adhesion molecules (ICAMs) (21). Structure and function analysis of the integrin β7 subunit has identified a small region (residues 46-386) that accounts for α4β7 binding specificity to its ligand, MAdCAM-1 (21). The α4β7 integrin is expressed on lymphocytes that are destined to migrate to mucosal surfaces, namely CD4+, CD8+ T cells, IgA+ plasma cells and plasmablasts (13, 22, 23).

Strategies for IBD Treatment

Conventionally, immunosuppressive agents that non-specifically control inflammation have been used to treat IBD (24, 25). Recently, therapies that involve generating mAbs against molecules specifically involved in the inflammatory cascade have been introduced (1). Infliximab, an anti-TNF-α mAb is currently used as therapy for CD (26). Other anti-cytokine mAbs that inhibit IFN-γ and IL-12 are still being investigated. Antibodies that inhibit molecules involved in the migration of leukocytes to the gut have also been developed. In animal models, mAbs against the β7 integrin subunit (FIB504) and MAdCAM-1 (MECA-367) were able to reduce inflammation in the mouse colon (27). For humans, Natalizumab is a humanized anti-α4 integrin mAb that is able to prevent α4β7/MAdCAM-1 interactions. There is one humanized mAb, MLN-02 (also known as ACT-1), that specifically recognizes human α4β7 (28). This mAb, however, is not

commercially available for research purposes. In this project, we attempt to produce a mouse mAb against the human α4β7 integrin to fulfill this need.

Research Goals

We adopted a novel immunization strategy to elicit a potent immune response against the β7 subunit of the α4β7 heterodimer. It involved immunizing β7 K/O mice with WT mouse splenocytes with naturally-occurring β7. The murine β7 subunit was chosen as the antigen because residues 46-386 of the murine and human β7 subunit (which account for α4β7 specificity for MAdCAM-1) are 92% homologous (21). This homology makes the murine β7 suitable for eliciting an immune response that can be boosted with human β7. The antibody produced would have a desired specificity to shared epitopes of mouse and human β7, in conformation with α4. Such an antibody could be used to test the hypothesis that IBD is associated with elevated blood levels of gut-homing lymphocytes, in particular plasmablasts. It would enable the tracking of these gut-specific plasmablasts in patient blood samples. Along with being linked to the disease state, plasmablast blood levels detected by such an antibody and other plasmablast markers could be used to monitor patient response to IBD therapy.

CHAPTER II

PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST THE ALPHA 4 BETA 7 INTEGRIN

PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST THE ALPHA 4 BETA 7 INTEGRIN[1]

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[3]Abbreviations used in this paper: mAb, monoclonal antibody; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; DC, dendritic cells; $\alpha 4\beta 7$, alpha 4 beta 7; MAdCAM-1, mucosal addressin cell adhesion molecule-1; $\beta 7$, beta 7; K/O, knock-out; WT, wild-type; PE, phycoerythrin; APC, allophycocyanin; PBMC, human peripheral blood mononuclear cells; CFA, complete Freund's adjuvant; SB, staining buffer; PEG, polyethylene glycol; CF, cloning factor; HAT, hypoxanthine, aminopterin, thymidine; HT, hypoxanthine, thymidine; pAb, polyclonal antibody; TNF-α, tumor necrosis factor alpha; RT, room temperature; CLA, cutaneous lymphocyte antigen

Abstract

This study describes the production of a murine monoclonal antibody (mAb^3) against the human alpha 4 beta 7 ($\alpha4\beta7$) gut-homing integrin receptor found on both T and B lymphocytes. The recruitment of lymphocytes via the $\alpha4\beta7$ receptor to the gut has been associated with chronic inflammatory bowel disease (IBD). There is currently only one anti-$\alpha4\beta7$ mAb in existence, but it is not commercially available. Studies involving this receptor are in need of such an antibody (Ab). A novel immunization strategy was employed in generating the desired polyclonal antibodies (pAb) in C57BL/6 mice. $\beta7$ knock-out (K/O) mice were immunized with $\beta7$ wild-type (WT) mouse splenocytes with naturally occurring cell-surface $\beta7$. As a final boost, mice were injected with human peripheral blood mononuclear cells (PBMC), with a rationale of boosting epitopes shared between murine and human $\beta7$. Immune sera and supernatants of several clones generated by hybridoma technology were tested using enzyme-linked immunosorbent assays (ELISA) and flow cytometry. In flow cytometric screening assays, the staining pattern of the Abs on PBMC expressing human $\beta7$ versus a mouse T cell line over-expressing $\alpha4\beta7$ molecules (TK1) was evaluated. Despite binding to both TK1 cells and PBMCs, the mAb produced is unlikely to be anti-$\alpha4\beta7$ because it generated a different staining pattern than the positive control, ACT-1, on T cell subsets that migrate to the gut.

Introduction

Inflammatory Bowel Disease

IBD is comprised of Crohn's disease (CD) and ulcerative colitis (UC), two chronic intestinal inflammatory conditions that currently affect about 1.4 million people in the United States (1). Although the etiology of IBD is still elusive, IBD is manifested by chronic mucosal inflammation that results from an aberrant immune response and an influx of inflammatory lymphocytes to the gut. This heightened mucosal immune reaction occurs in response to harmless bacterial antigens (2).

In healthy individuals, the immune system is able to distinguish between commensal bacteria that maintain a symbiotic relationship with their hosts and pathogens (3). In IBD however, DCs falsely respond to indigenous microbial antigens and mature, inducing effector T cell and pro-inflammatory responses (4). Pro-inflammatory cytokines such as IL-12, IL-18, IL-23, tumor necrosis factor alpha (TNF-α) and IFN γ are released and up-regulate the expression of adhesion molecule ligands on the vascular endothelium encouraging leukocyte adhesion and extravasation into the tissue (5-7). T and B lymphocytes and macrophages are among the leukocytes that respond to these chemotactic signals. Upon reaching the antigen site, macrophages then release chemokines that accumulate at endothelial cell surfaces and attract more inflammatory leukocytes, continuing the cycle. An interplay of all these immunologic events leads to perpetual inflammation in what is known as IBD (2).

The recruitment process of lymphocytes to the gut involves several sequential steps. The cells are in transit, under high flow conditions in the bloodstream and must come to an

arrest and proceed through the vasculature to the tissue of interest (8, 9). Primary adhesion which is transient and reversible is accomplished by selectins such as L-selectin found on lymphocytes binding their ligands on the endothelial cell surface (10). This interaction allows for loose rolling of the lymphocytes along the vessel wall and successfully slows their transit. Lymphocytes are then activated by an interaction between chemokines generated on the endothelial surface such as CCL25 and chemokine receptors such as CCR9 found on the cells (11, 12). This interaction increases the affinity of integrin receptors for their adhesion molecules and promotes their binding. Specifically, the $\alpha 4\beta 7$ integrin on the lymphocyte cell-surface binds the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) found on the endothelium. This results in an activation-dependent arrest and firmly adheres the lymphocytes to the endothelium. Finally, cells undergo transmigration, the passage of lymphocytes through intact vessel walls to the surrounding tissue (13-15).

Structure and function analysis of the integrin $\beta 7$ subunit of the $\alpha 4\beta 7$ heterodimer identified a small region (residues 46-386) that accounts for $\alpha 4\beta 7$ binding specificity to its ligand, MAdCAM-1 (16). The $\alpha 4\beta 7$ integrin is expressed on lymphocytes that are destined to migrate to mucosal surfaces, namely CD4+, CD8+ T cells, IgA+ plasma cells and plasmablasts (9, 17, 18).

In order to study $\alpha 4\beta 7$ in association with IBD, an attempt was made to generate a mAb against the integrin. A novel immunization strategy was adopted for eliciting a potent immune response against the $\beta 7$ subunit of the $\alpha 4\beta 7$ heterodimer. It involved immunizing $\beta 7$ K/O mice with WT mouse splenocytes with naturally-occurring $\beta 7$. The

murine β7 subunit was chosen as the antigen because residues 46-386 of the murine and human β7 subunit (which account for α4β7 specificity for MAdCAM-1) are 92% homologous (16). This homology makes the murine β7 suitable for eliciting an immune response that can be boosted with human β7. The antibody produced would preferentially be against shared epitopes of mouse and human β7, in conformation with α4.

The purpose of such an antibody was to test the hypothesis that IBD is associated with elevated blood levels of gut-homing lymphocytes, in particular plasmablasts. It would enable the tracking of these gut-specific plasmablasts in patient blood samples. Along with being linked to the disease state, plasmablast blood levels detected by such an antibody and other plasmablast markers could be used to monitor patient response to IBD therapy.

Materials and Methods

All studies using animal and human subjects have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) (Protocol #904) and Human Subjects-Institutional Review Board (IRB) (Protocol #F0902030) at San José State University, San José, CA.

Cells and media

TK1 is a CD8+ T lymphoma cell line over-expressing α4β7 that originated from a spontaneous thymic lymphoma in an AKR/Cum mouse [American Type Culture Collection (ATCC) CRL-2396] (a gift of Eugene Butcher's, Stanford University). Cryopreserved PBMC were also a gift from Dr. Butcher's lab. They were isolated by a Ficoll gradient. Sp2/O myeloma cells were obtained from San José State University. All cells were stored in liquid nitrogen until needed, then maintained in culture in supplemented RPMI 1640 [2 mM L-glutamine (Lonza), 10% fetal calf serum (FCS) (Invitrogen), penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml)/ amphotericin B (25 μg/ml) (ThermoScientific), 1 mM sodium pyruvate (ThermoScientific) and 1 mM non-essential amino acids (NEAA) (Lonza)]. Fresh splenocytes for screening assays and immunizations were aseptically removed from female C57BL/6 β7 WT and/or β7 K/O mice via splenectomies. Cells were prepared by pressing spleens through a 40 μm nylon mesh strainer (BD Biosciences) while washing with supplemented RPMI 1640. Red blood cells in the mixture were lysed with 3 ml of red blood cell lysis buffer (8.3 g/L ammonium chloride in 0.01M Tris-HCl buffer, pH 7.5) (Sigma) for 3 min. The remaining cells were then washed twice with phosphate buffered saline (PBS) and

counted. Feeder layer cells (peritoneal macrophages) were obtained by injecting 10 ml of supplemented RPMI 1640 into the peritoneal cavity of a BALB/c mouse with a 22 gauge needle and 10 ml syringe, massaging the abdomen and then withdrawing the fluid.

Immunizations

Three 6 wk old female C57BL/6 β7 K/O mice (identified as Mouse L, Mouse LR and Mouse R) were first immunized subcutaneously (s.c.) with an emulsion of 200 µl of complete Freund's adjuvant (CFA) (Sigma) and 100 X 10^6 β7 WT mouse splenocytes. Subsequently, mice were immunized s.c. every 2 wks for approximately 8 wks with an emulsion of 200 µl of incomplete Freund's adjuvant (IFA) (Sigma) and an average of 70 X 10^6 cells per mouse. Approximately 2 wks after the last immunization with β7 WT splenocytes, mice were bled and sera were tested by ELISA and flow cytometry assays to assess pAb titers. Mice received their last immunization intravenously or intraperitoneally with 60 X 10^6 PBMC (thawed and washed once with PBS) in 200 µl PBS, 3-4 days before fusion. A flow-chart summary of the immunization strategy is shown in Fig. 2.

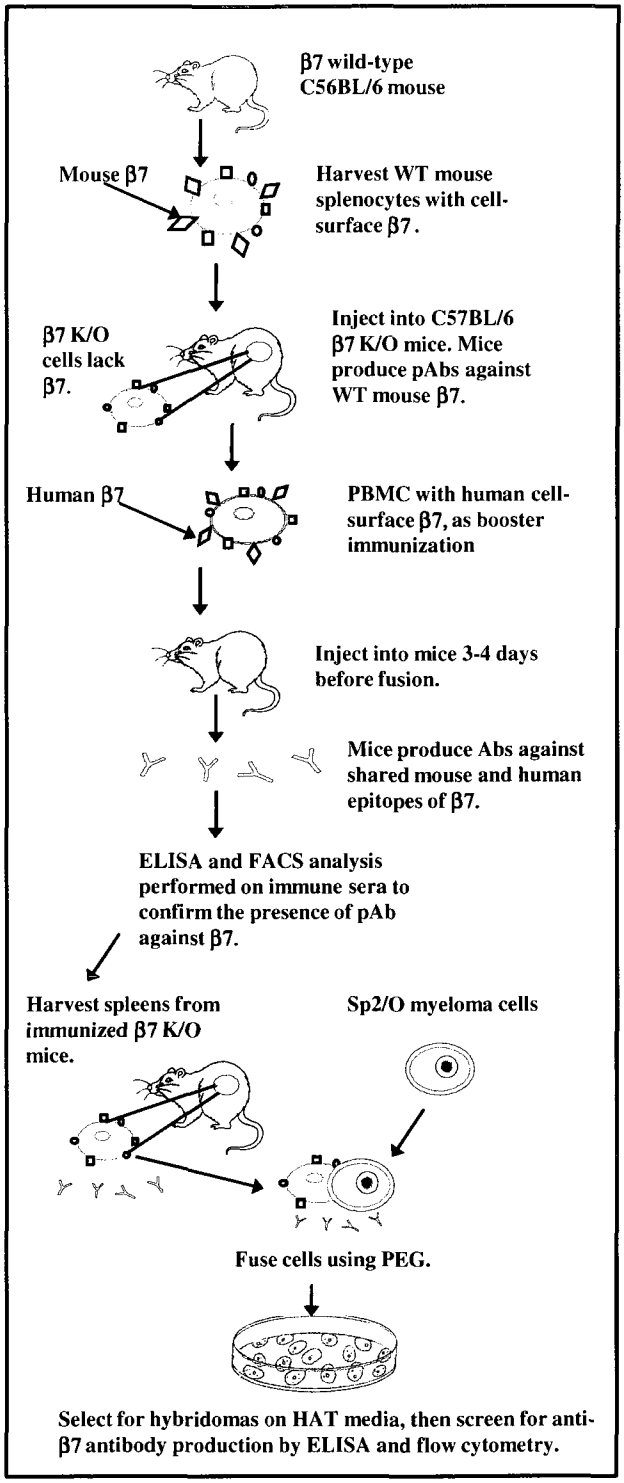

FIGURE 2. Flow-chart summary of the immunization strategy.

Ab titration using ELISA and flow cytometry

ELISA: An indirect cell-based ELISA was performed on U-bottom 96-well plates (Costar). One to five hundred thousand TK1 cells were seeded per well and blocked at room temperature (RT) for 40 min with 150 µl/well blocking buffer (2% FCS, 0.05% Tween-20 in PBS). Plates were then centrifuged (300 X *g* for 5 min), decanted and the cells resuspended in 100 µl/well blocking buffer supplemented with 5% goat serum, followed by incubation at RT for another 20 min. Plates were then centrifuged, decanted and cells were resuspended in 100 µl/well primary Ab [negative control (anti-*Listeria monocytogenes* pAb), preimmune and immune sera] in blocking buffer. Two-fold serial dilutions of each of the primary Abs were made starting with 1/50, followed by incubation at 4°C for 1 h. Cell pellets were disrupted by gently shaking plates every 15 min on a plate shaker (ThermoScientific) for 30 s. After the incubation, cells were washed 3 times with 150 µl/well blocking buffer. One hundred microliters per well of alkaline phosphatase-conjugated secondary Ab (goat anti-mouse IgG, whole molecule) (Sigma) (1/1000) diluted in blocking buffer was added and cells were incubated for at 4°C for 1 h. Cells were washed 2 times with 150 µl/well of blocking buffer and a third time with PBS. Plates were decanted and developed with 175 µl/well of alkaline-phosphatase substrate (1.0 mg/ml p-Nitrophenyl phosphate and 0.2 M Tris buffer) (Sigma) dissolved according to the manufacturer's instructions. Thirty to forty-five min later when color had developed, absorbance was determined at 405 nm.

Flow cytometry analysis: One million TK1 cells/tube were kept on ice and blocked with a 1/50 dilution of goat serum in staining buffer (SB) [1% FCS, 0.1% sodium azide (Sigma) in PBS] and incubated for 10 min at 4°C. Cells were centrifuged (300 X *g*, 5 min), resuspended with a mixture of goat serum (1/50) and two-fold serial dilutions of primary Abs [negative control (anti-*Listeria monocytogenes* pAb), positive control (ACT-1, mouse anti-human α4β7; FIB504, rat anti-mouse β7), preimmune and immune sera] and incubated at 4°C for 30 min. Hybridoma supernatants of the positive control Abs were a gift from Dr. Butcher's lab, Stanford University. Cells were then washed 3 times with SB and resuspended with secondary-labeled Ab, goat anti-mouse IgG or goat anti-rat IgG conjugated to phycoerythrin (PE) (Jackson ImmunoResearch) and incubated in the dark at 4°C for 30 min. Tubes were then washed 3 times with SB. Cells were fixed in 500 µl/tube of 1% formaldehyde (formalin diluted in PBS) (Sigma) and analyzed by flow cytometry (FACSCalibur; BD Biosciences). Ten thousand events were acquired in all flow cytometry experiments. Quadrants on dot plots in all flow cytometry experiments were set according to the secondary-labeled Ab control. The FL2 fluorescence detector in the cytometer is able to detect PE which emits a fluorescence signal near 585 nm.

Polyclonal Ab sera assessment for β7 specificity

Immune sera were assessed by FACS analysis for β7 specificity using a mixture of TK1 cells and PBMC, 5 X 10^5 each per tube. Frozen PBMC were thawed, washed once with RPMI 1640 to remove DMSO and counted. Staining of PBMC was performed as described above for TK1 cells except 1/200 dilutions of preimmune and immune sera

were used as primary Abs. Sera were also tested by flow cytometry on β7 WT and β7 K/O splenocytes. Splenocytes were prepared as described above. Two identical experiments were run in parallel with the two types of splenocytes. The assay was also identical to the one described above for TK1 cells except for some additional steps. A 1/200 dilution of each mouse serum was used. After washing to remove unbound secondary Ab, cells were blocked with a mixture of rat and mouse sera (eBioscience and Harlan Bioproducts, respectively) diluted 1/50 and incubated at 4°C in the dark for 10 min. Cells were washed three times and a mixture of rat serum (1/50), mouse serum (1/50) and a 1/200 dilution of rat anti-mouse CD45R/B220 Ab conjugated to FITC (BD Pharmingen) was added. Anti-B220 Ab was used to exclude B cells. After a 20 min incubation at 4°C in the dark, cells were washed as before, resuspended in 500 µl/tube of 1% formaldehyde and analyzed on the FACSCalibur. FITC is detected by the FL1 fluorescence detector in the cytometer which detects fluorescence signals near 530 nm.

Fusion

Three separate fusions were performed using splenocytes from the 3 immunized mice (L, LR and R). Three to four days after the final booster, the spleen was removed and processed as described above. Healthy Sp2/O myeloma cells in log phase of growth were mixed with splenocytes at a ratio of 1:3. After centrifugation, 2 ml of 50% polyethylene glycol (PEG) (Sigma) in plain RPMI-1640 (lacks FCS, sodium pyruvate and NEAA) was slowly added to the cell mixture pellet over 1 minute, followed by 2 ml of plain RPMI-1640 (over 5 min) and 20 ml of plain RPMI-1640 slowly but not drop-wise. Cells were centrifuged and resuspended in RPMI-1640 supplemented with 20%

FCS and 10% hybridoma cloning factor (CF) (BioVeris Corp) to obtain a concentration of 1.3 X 10^6 cells/ml. Five different concentrations of fused cells (1 X 10^5, 5 X 10^4, 2.5 X 10^4, 1 X 10^4 and 5 X 10^3 cells/well) resuspended in supplemented RPMI (150 µl/well final volume) were co-cultured in 96-well flat-bottom plates (Corning Costar 3596) with 4 X 10^3 cells/well of peritoneal macrophages (as feeder cells). The day after the fusion, 50 µl/well of 4X HAT medium [hypoxanthine, aminopterin and thymidine (HAT) supplement (MP BioMedicals) mixed with RPMI-1640 with 2 mM L-glutamine, 20% FCS, penicillin (10,000 U/ml)/ streptomycin (10,000 µg/ml)/ amphotericin B (25 µg/ml), 1 mM sodium pyruvate, 1 mM NEAA and 50 µM β–mercaptoethanol] was added to the cells to generate 1X HAT.

Hybridoma screening

Hybridomas were microscopically screened 10-12 days post-fusion. Supernatants from single clones occupying at least one third of the bottom of the well were tested by ELISA and flow cytometry as described above, using TK1 cells for the ELISA and a mixture of TK1 cells and PBMC for the flow cytometric assays. Positive clones (those that had atleast 1.6 times higher absorbance values than the preimmune serum for the ELISA and considerably brighter staining in flow cytometry) were subcloned by the limiting dilution method.

Limiting dilution

Twenty-five microliters of resuspended hybridoma cells in supplemented HAT media were removed from the positive clone wells and added to a well on a new 96-well plate containing 100 µl of 1X HT media (Mediatech) (prepared the same as the HAT

media except aminopterin was excluded). Sequential dilutions (1/5) were performed for a few additional wells containing 100 µl of HT media. The contents of a well with an average of 25 cells (counted microscopically) were transferred to 40 ml of HT media (with 5% CF), mixed and plated onto three 96-well flat-bottom plates (100 µl/well in two plates and 200 µl/well in the third plate).

Flow cytometry analysis of subclones for β7 Specificity

Subclones from the original positive hybridomas were analyzed for β7 specificity with flow cytometry using screening assays as described above. They were tested on a mixture of PBMC and TK1 cells and on β7 WT and β7 K/O splenocytes. Positive clones were cryopreserved at -80°C for 2 days and then transferred to liquid nitrogen. One to two million cells per vial were frozen in 1 ml of freezing media (50% RPMI-1640, 40% FCS, 10% DMSO).

Competition assays

Positive clones were analyzed in competition assays to see if the Abs they produced could block positive control Abs from binding TK1 cells or PBMC. One million TK1 cells/tube were first exposed for 30 min to primary Abs, washed and then cells were incubated with 50 µl of FIB504 for 30 min. The cells were washed again 3 times and resuspended in 100 µl of a 1/100 dilution of goat anti-rat secondary Ab conjugated to PE and incubated at 4°C for 30 min. Cells were then fixed with 1% formaldehyde and analyzed by flow cytometry. For experiments using PBMC, 1 X 10^6 PBMC/tube were blocked with 0.5 µl of goat serum for 10 min. After centrifugation (300 X *g*, 5 min), 50 µl of primary Abs [FIB504 positive control Ab (supernatant) or

preimmune or immune sera (diluted 1/200 in SB) or hybridoma supernatants] were added to the cells and incubated at 4°C for 30 min. Cells were then washed and stained with 5 µl of ACT-1 conjugated to allophycocyanin (APC) (a gift of Eugene Butcher's, Stanford University) together with 10 µl of mouse blocking serum. They were allowed to incubate for 20 min at 4°C in the dark and then washed. Finally, the cells were resuspended in 500 µl/tube of 1% formaldehyde and acquired by flow cytometry. The FL4 fluorescence detector in the cytometer is able to detect APC which emits a fluorescence signal near 675 nm.

Multi-color assay

A multi-color FACS analysis was performed on human PBMC. Hybridoma subclone supernatants and the polyclonal Ab serum were used to stain PBMC and their staining pattern was compared with the staining pattern generated by the human anti-$\alpha 4\beta 7$ antibody (ACT-1). One million PBMC were blocked with 0.5 µl goat serum for 10 min. Fifty microliters of primary Ab was added to tubes with cells and incubated at 4° C for 30 min. Cells were washed with 3 ml of SB then resuspended with goat anti-mouse secondary Ab conjugated to PE. They were incubated for 30 min (in the dark) at 4° C then washed again with 3 ml of SB. A master mix composed of 5 µl/tube anti-CD45RA FITC, 10 µl/tube biotinylated anti-cutaneous lymphocyte antigen (CLA), 1 µl/tube anti-CD4 APC and 5 µl/tube anti-CD62L Cy7 APC was made and added to the cells to isolate the desired subset of cells. After a 20 min incubation at 4° C (in the dark), cells were washed again with 3 ml of SB. Finally, 1.5 µl/tube streptavidin Cy7 PE were added to the tubes that contained biotinylated primary Abs and incubated for another 20 min.

After a final 3 ml wash, cells were resuspended with 500 µl/tube SB and analyzed on a BD FACSAria flow cytometer (at the VA hospital, Palo Alto/Stanford University flow cytometry facility).

Results

EVALUATION OF POLYCLONAL AB SERA

Assessment of pAb titers by ELISA and flow cytometry using β7–rich TK1 cells

β7 K/O mice were immunized with β7 WT mouse splenocytes. Immune sera from the mice were tested during the immunization process for pAb titer on TK1 cells using two methods: ELISA and flow cytometry (Fig. 3). Absorbance values for the immune serum dilutions (red bars) were higher than the preimmune serum dilutions (green bars) (Fig. 3A), indicating an immune response against the β7 found on WT splenocytes. In flow cytometry experiments, three dilutions of polyclonal serum, 1/50 and 1/100 (orange peak) and 1/200 (blue peak) all bound β7 found on TK1 cells, as reflected by the shift in the cells' fluorescence intensity. In comparison, the preimmune serum (brown peak), secondary Ab control (purple peak) and negative control (irrelavant pAb) (green peak) (Fig. 3B) did not bind TK1 cells.

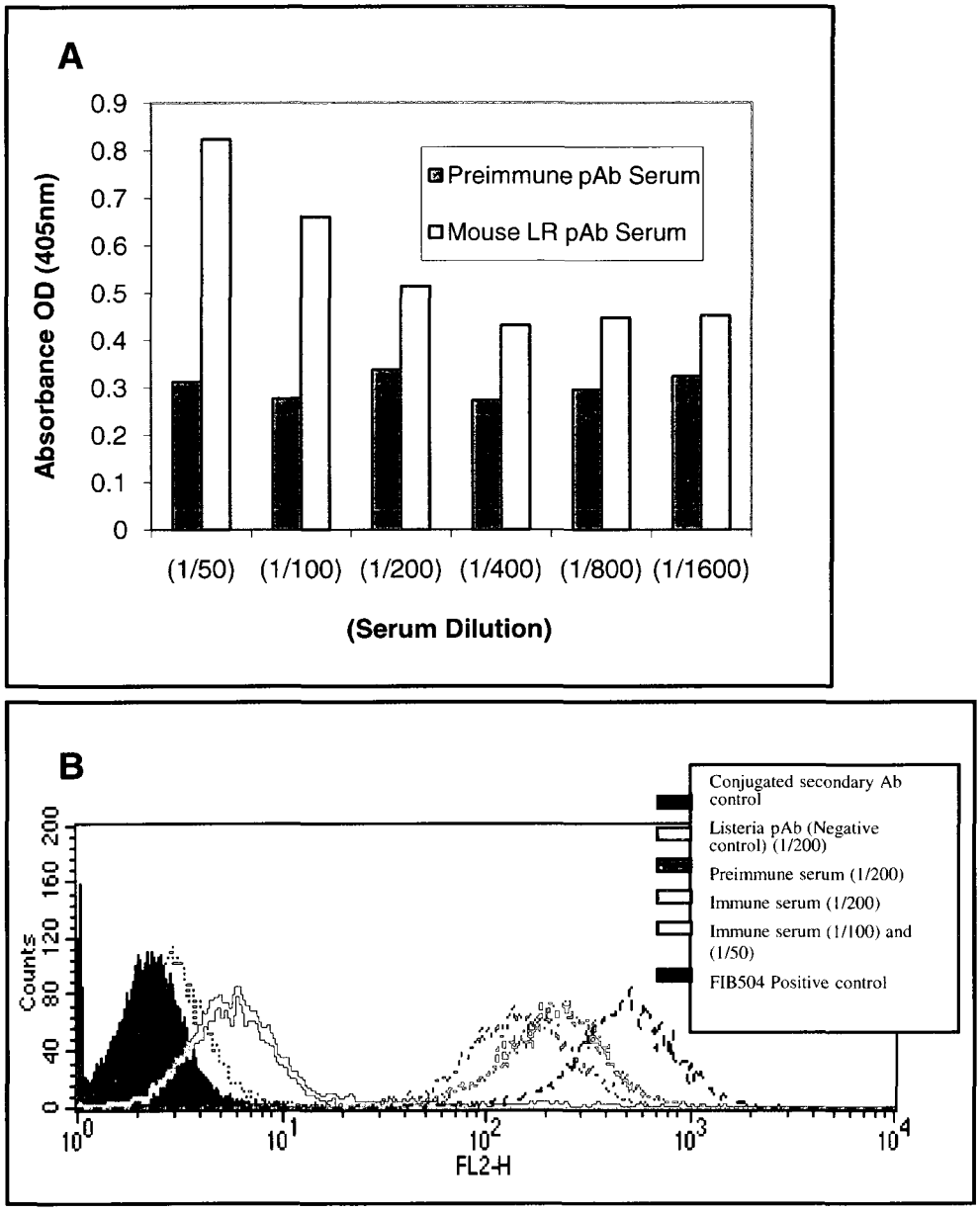

FIGURE 3. pAb titer assessment using β7-rich TK1 cells. A. ELISA of pre- and post-immune sera from mice immunized with β7 WT splenocytes. Plates were coated with TK1 cells. B. Flow cytometry analysis of pAb sera from mice immunized with β7 WT splenocytes. pAb binding was tested on TK1 cells. PE is conjugated to goat anti-mouse secondary Abs (FL2-H). Results are representative of similar results obtained with sera from all three mice.

Specificity of pAb sera for β7 WT splenocytes versus β7 K/O splenocytes

The specificity of the immune sera was further examined by testing on β7 WT mouse splenocytes (left column) versus β7 K/O mouse splenocytes (right column) (Fig. 4C). Sera that are specific for β7 should not bind β7 K/O splenocytes lacking the β7 molecule. B cells were excluded from the analysis using anti-B220 Abs conjugated to FITC, since cell-surface Abs (IgM and IgD) and Fc receptors found on B cells are known to be responsible for non-specific binding. The preimmune serum sample bound only ~6 % of β7 WT cells. However, a significant percentage of β7 WT cells stained with the pAb sera shifted to higher fluorescence values (36.5% for Mouse R, 34.9% for Mouse L and 56.6% for Mouse LR), representing the sera's ability to recognize and bind β7. On the other hand, there was a significant reduction in β7 K/O cell binding compared to the β7 WT cell binding by all three pAb sera. The small percentage of β7 K/O cells that were stained (13.0% for Mouse R, 10.3% for Mouse L and 15.0% for Mouse LR) is suspected to be non-specific binding or the presence of pAb to non-β7 molecules. Non-specific binding of the sera can be subtracted resulting in 23.5% of β7 WT cells bound by Mouse R serum, 24.6% of β7 WT cells bound by Mouse L serum and 41.6% of β7 WT cells bound by Mouse LR serum. As expected, the positive control did not bind β7 K/O cells. This assay suggests that the differential binding of the immune pAb to WT splenocytes but not to K/O splenocytes reflects the pAb sera binding to β7 molecules.

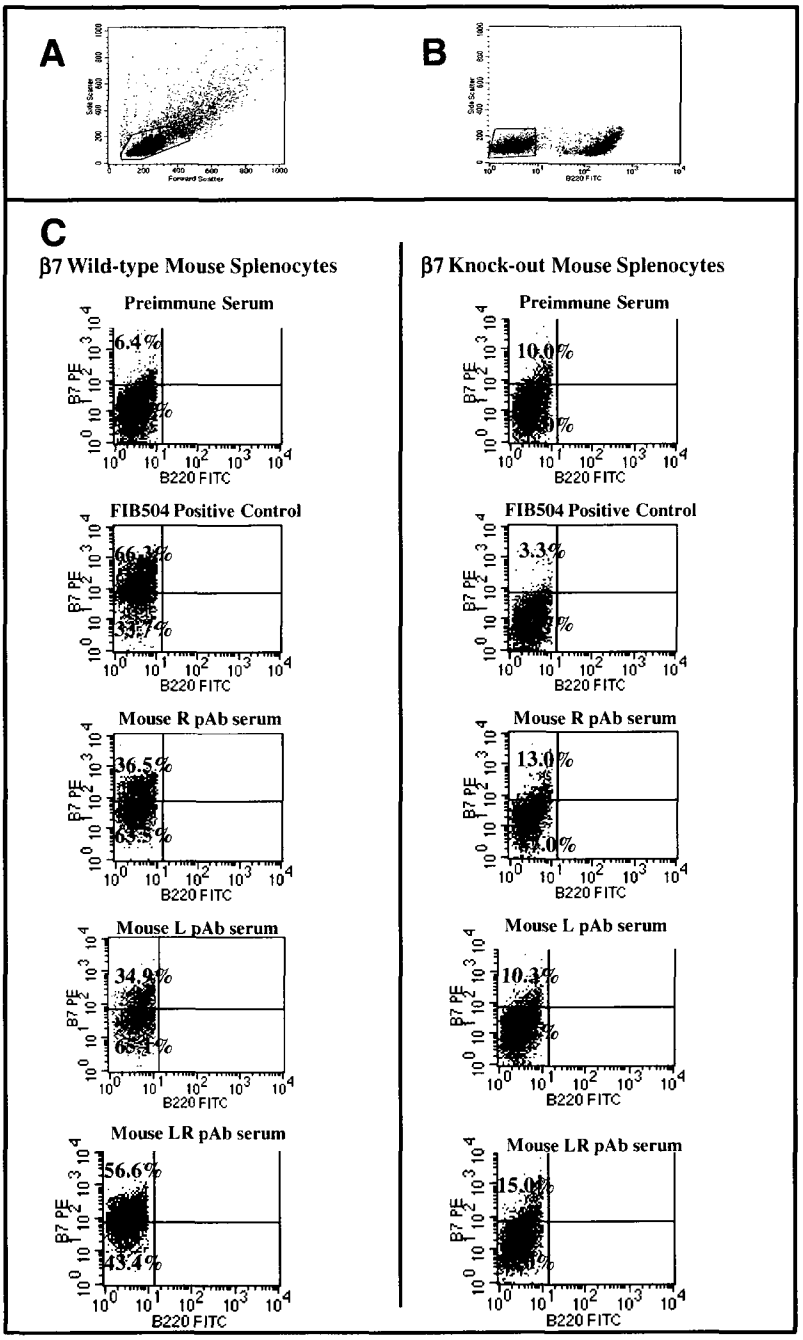

FIGURE 4. pAb sera screening on β7 WT versus β7 K/O mouse splenocytes. A. Unstained splenocytes, gate R2 includes presumed mononuclear cells. B. The dot plot is gated on R2, region R1 has mononuclear cells unstained by anti-B220 antibodies conjugated to FITC. All panels in (C) are gated on R1 and R2, excluding B cells. PE is conjugated to goat anti-mouse secondary Abs (FL2-H).

Determination of immune sera specificity for mouse and human β7

To verify that there was an immune response against human β7 as well as mouse β7 following the booster with PBMC, flow cytometric assays using a mixture of PBMC and TK1 cells were performed (Fig. 5). TK1 cells can be distinguished from PBMC on forward scatter based on size since PBMC are significantly smaller than the TK1 cancer cells (Fig. 5A). The preimmune serum, secondary Ab control and negative control (*Listeria monocytogenes* pAb) only nonspecifically bound both types of cells, with an average of 6% of TK1 cells and 7% of PBMC being bound for the three mice. The pAb sera from all three mice bound β7 on the human cells as well as the TK1 cells. The sera from Mouse LR, R and L bound 37.6%, 40.6% and 77.4% of the human cells and 93.4%, 91.6% and 96.7% of TK1 cells, respectively (Fig. 5B). Two positive controls were used in this experiment, FIB504 which is a rat anti-β7 mAb that binds both mouse and human cells and ACT-1, a mouse anti-α4β7 mAb that exclusively binds human cells. Results indicate that both positive controls behaved as expected and that polyclonal sera Abs were able to recognize and bind human β7 as well as mouse β7. Flow cytometry was preferred over ELISA for future screening experiments because two cell types (such as TK1 cells and PBMC) can be mixed and distinguished in flow cytometry simultaneously generating information about the sera or supernatants' specificity for both mouse and human β7.

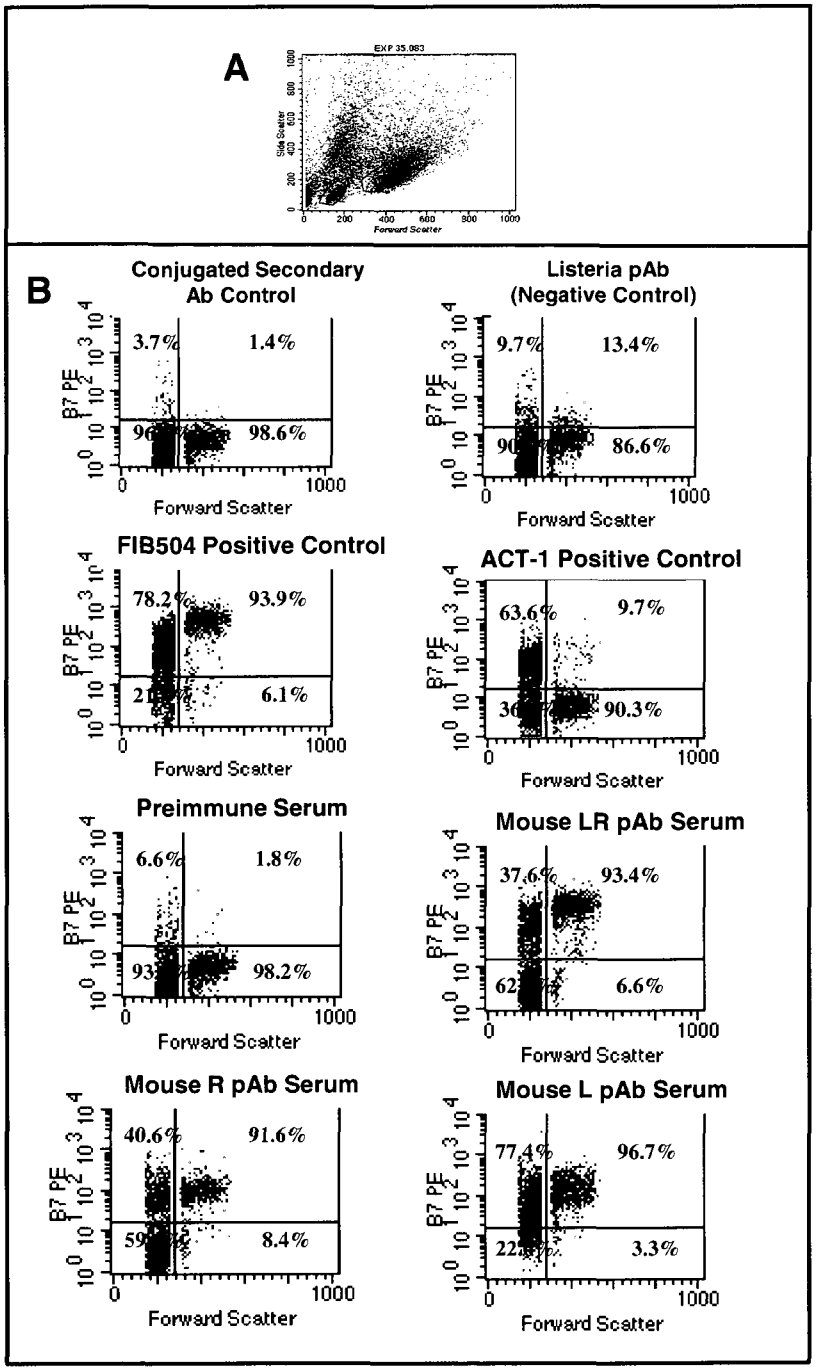

FIGURE 5. Flow cytometric screening of pAb sera on mixed TK1 and PBMC populations. A. The dot plot shows the regions for PBMCs (green, R2) and TK1 cells (red, R1). B. Dot plots are gated on R1 or R2. PE is conjugated to goat anti-mouse secondary Abs (FL2-H).

HYBRIDOMA YIELD FOLLOWING FUSION

Three separate fusions were performed sequentially based on the highest pAb titer of the mouse sera, assessed by several of the experiments described previously. All three fusions were successful in producing hybridomas. Approximately 795 hybridomas were obtained from three fusions. Mouse L generated 218 clones while Mouse LR and Mouse R produced 121 and 456 clones, respectively (Table 1).

Table 1. Summary of hybridoma yield following fusion

Mouse	Total Hybridomas	Single Clones	% Single Clones	Original Positive Hybridomas	Subcloned
L	218	115	53%	14	13
LR	121	97	80%	22	11
R	456	302	66%	77	16

SPECIFICITY OF HYBRIDOMA SUPERNATANTS FOR MOUSE AND HUMAN β7

Hybridomas were initially screened using ELISA (data not shown) and positive supernatants were used to stain a mixture of PBMC and TK1 cells in flow cytometric assays to determine specificity for β7. A majority of the hybridomas were negative for anti–β7 Ab. However, Mouse L produced 14 hybridomas that responded to the β7 antigen. Mouse LR and Mouse R produced 22 and 77 positive hybridomas, respectively (Table 1). A few of the positive hybridomas exhibiting different staining patterns, G5-H5, F9-D3, D6-E12, B7-E8 and F4-A9 are depicted in Fig. 6 along with an example of a negative hybridoma. Several of the positive hybridomas were subcloned by limiting dilution.

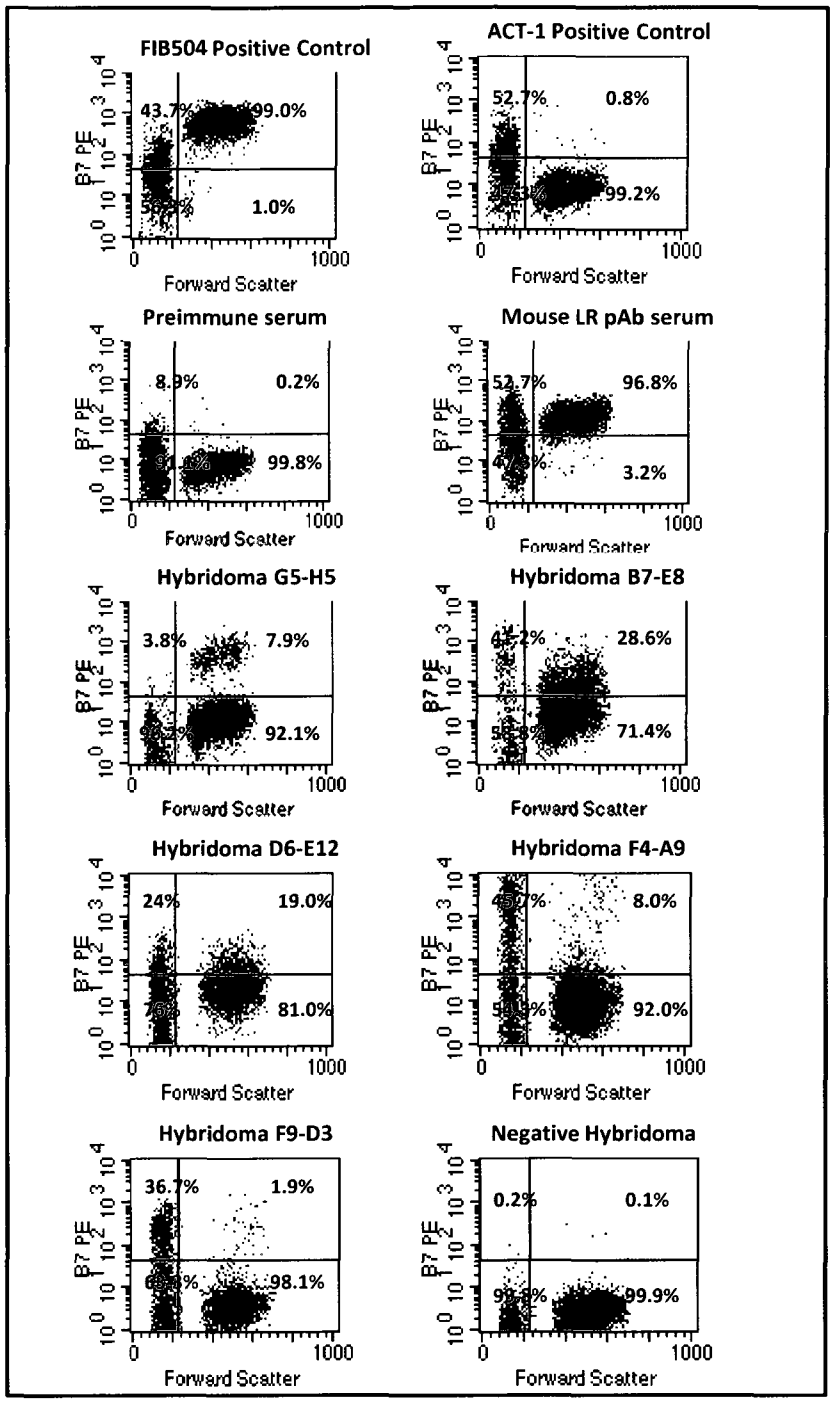

FIGURE 6. Flow cytometric screening of original hybridoma supernatants on mixed TK1 and PBMC populations. Dot plots are gated on TK1 cells (red) and PBMC (green) populations, R2 or R1, respectively (not shown). PE is conjugated to goat anti-mouse secondary Abs (FL2-H).

Subclones were retested by flow cytometry and many showed staining patterns similar to those generated by the original hybridomas. Those that remained positive (subclones of hybridoma G5-H5, F9-D3, D6-E12, B7-E8 and F4-A9) are examined in Fig. 7. Judging from their staining patterns, G5-H5 is an example of a clone that binds TK1 cells only and not PBMC while clone F9-D3 shows the opposite by binding only PBMC. D6-E12 is a clone that binds both types of cells moderately well. Furthermore, clones B7-E8 and F4-A9 seemed to produce Abs that recognized β7 well on both types of cells. Subclone F4-A9 showed higher specificity for the TK1 cells than the original F4-A9 hybridoma. An example of a negative subclone from hybridoma F4-A9 that did not produce anti-β7 Ab is also shown.

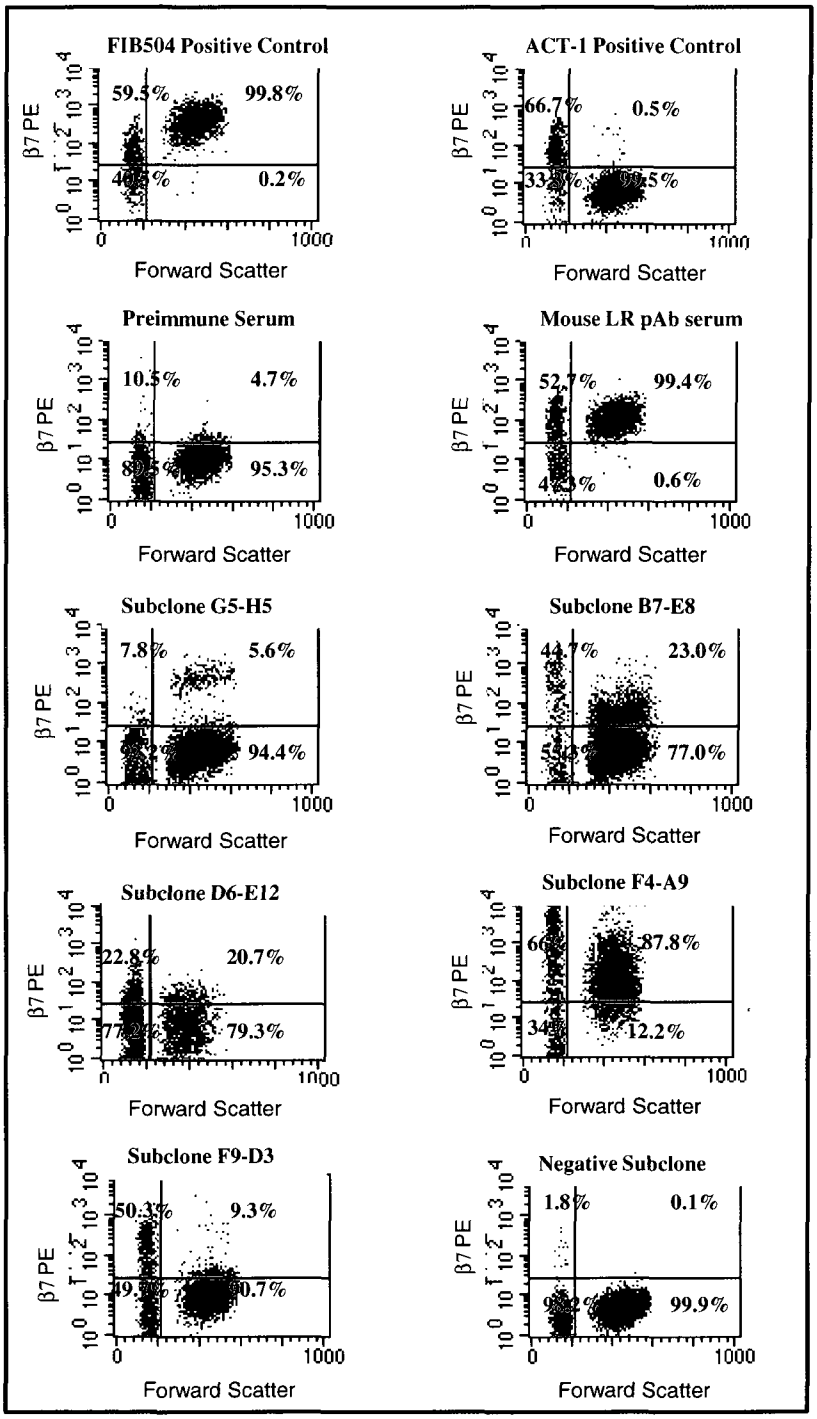

FIGURE 7. Flow cytometric screening of hybridoma subclone supernatants on mixed TK1 and PBMC populations. Dot plots are gated on TK1 cells (red) and PBMC (green) populations, R2 or R1, respectively (not shown). PE is conjugated to goat anti-mouse secondary Abs (FL2-H).

To further investigate the specificity of these clones for β7, supernatants were also evaluated on β7 WT splenocytes versus β7 K/O mouse splenocytes like the sera (Table 2). None of the supernatants bound well to the β7 WT cells (represented by mean fluorescence values in the FL2 channel). The pAb serum also did not respond to the β7 WT splenocytes as well as it did in previous experiments. Only the positive control FIB504 bound the β7 WT cells. Furthermore, there was no significant reduction in the mean fluorescence when the supernatants were applied to β7 K/O cells. The positive control solely displayed a reduction in the mean fluorescence values when bound to β7 K/O cells versus being bound to β7 WT cells.

Table 2. Hybridoma subclone supernatant screening on β7 WT versus β7 K/O mouse splenocytes[a]

Primary Antibody	**β7 WT Splenocytes FL2 (PE) Arithmetic Mean Fluorescence (Arbitrary Units)**	**β7 K/O Splenocytes FL2 (PE) Arithmetic Mean Fluorescence (Arbitrary Units)**
Listeria pAb (Negative Control)	8.89	16.77
FIB504 Positive Control	48.45	9.41
Preimmune Serum	13.29	16.50
Mouse R pAb Serum	28.43	24.40
Subclone G5-H5 supernatant	9.45	17.04
Subclone B7-E8 supernatant	8.89	17.19
Subclone D6-E12 supernatant	13.41	16.53
Subclone F4-A9 supernatant	33.09	40.23
Subclone F9-D3 supernatant	15.53	15.35

[a] Binding of anti-β7 Ab found in the subclone supernatants to splenocytes is represented by mean fluorescence values (arbitrary units) in the FL2 channel. Goat anti-mouse secondary Abs are conjugated to PE. B cells were excluded from the flow cytometry analysis with anti-B220 Abs conjugated to FITC.

COMPETITION ASSAYS TO ASSESS AB FUNCTION

In order to assess if the Abs produced by these clones were blocking Abs able to block the positive controls FIB504 (Table 3) and/or ACT-1 (Table 4) from binding β7, competition assays were performed. The positive control samples were not blocked by primary Ab. Neither the polyclonal serum nor the supernatants were able to block the FIB504 Ab from binding β7 on the TK1 cells, since no reduction in mean fluorescence can be detected compared to the positive control (Table 3). In the competition assay using ACT-1 Ab, only the FIB504 was able to block the Ab from binding β7 on the PBMC. This is evident from the reduction of the mean fluorescence in the FIB504 sample. The polyclonal serum as well as the supernatants did not block the ACT-1 Ab, reflected by the mean fluorescence values remaining consistently high (Table 4). The results of both competition assays suggest that none of the clones produce blocking Abs.

Table 3. FIB504 competition assay to assess the subclone supernatant Abs' ability to block FIB504 from binding β7 on TK1 cells[a]

Primary Antibody	**TK1 cells FL2 (PE) Arithmetic Mean Fluorescence (Arbitrary Units)**
FIB504 Positive Control	756.39
Listeria pAb (Negative Control)	734.41
Preimmune Serum	719.87
Mouse LR pAb Serum	659.34
Subclone G5-H5 supernatant	751.82
Subclone B7-E8 supernatant	735.63
Subclone D6-E12 supernatant	749.28
Subclone F4-A9 supernatant	768.43
Subclone F9-D3 supernatant	750.05

[a] Mean fluorescence values (arbitrary units) represent goat anti-rat secondary Abs (conjugated to PE) binding rat-anti mouse β7 (FIB504).

Table 4. ACT-1 competition assay to assess the subclone supernatant Abs' ability to block ACT-1 from binding α4β7 on PBMC[a]

Primary Antibody	**PBMC FL4 (APC) Arithmetic Mean Fluorescence (Arbitrary Units)**
ACT-1 Positive Control	35.54
FIB504 Positive Control	11.21
Listeria pAb (Negative Control)	34.91
Preimmune Serum	34.79
Mouse LR pAb Serum	33.01
Subclone G5-H5 supernatant	33.93
Subclone B7-E8 supernatant	36.62
Subclone D6-E12 supernatant	35.42
Subclone F4-A9 supernatant	32.06
Subclone F9-D3 supernatant	35.57

[a] Mean fluorescence values (arbitrary units) represent anti-α4β7 ACT-1 (directly conjugated to APC) binding PBMC.

MULTI-COLOR ASSAY TO ASSESS AB BINDING TO A SUBSET OF T CELLS

Finally, the supernatants combined with additional multi-color antibodies were used to stain PBMC, focusing on CD4+ effector memory T helper cells that migrate to the gut. These cells are also $CD45RA^{low}$ $CD62L^{low}$ and have suppressed levels of CLA. The staining patterns of subclone supernatants were compared with the staining pattern generated by the ACT-1 positive control (Fig. 8, C and D). The supernatants' ability to bind this specific type of T cell subset and generate a pattern similar to that of the positive control ACT-1 would indicate the presence of anti-$\alpha 4\beta 7$ antibodies that recognize the integrin. Compared to the ACT-1 Ab however, the Abs found in the subclone supernatants and the polyclonal Ab serum generated different staining patterns on effector memory T cells (Fig. 8, C and D). The ACT-1 showed three distinct populations indicating the presence of cell populations that specifically migrate to the gut, the skin or neither (Fig.8D). However, both the polyclonal Ab serum and subclone supernatants only revealed two distinct populations: one that was CLA negative and presumably $\beta 7$ positive and another which was positive for both CLA and $\beta 7$, which is unlikely because effector memory T cells would migrate to one or the other destination, not both.

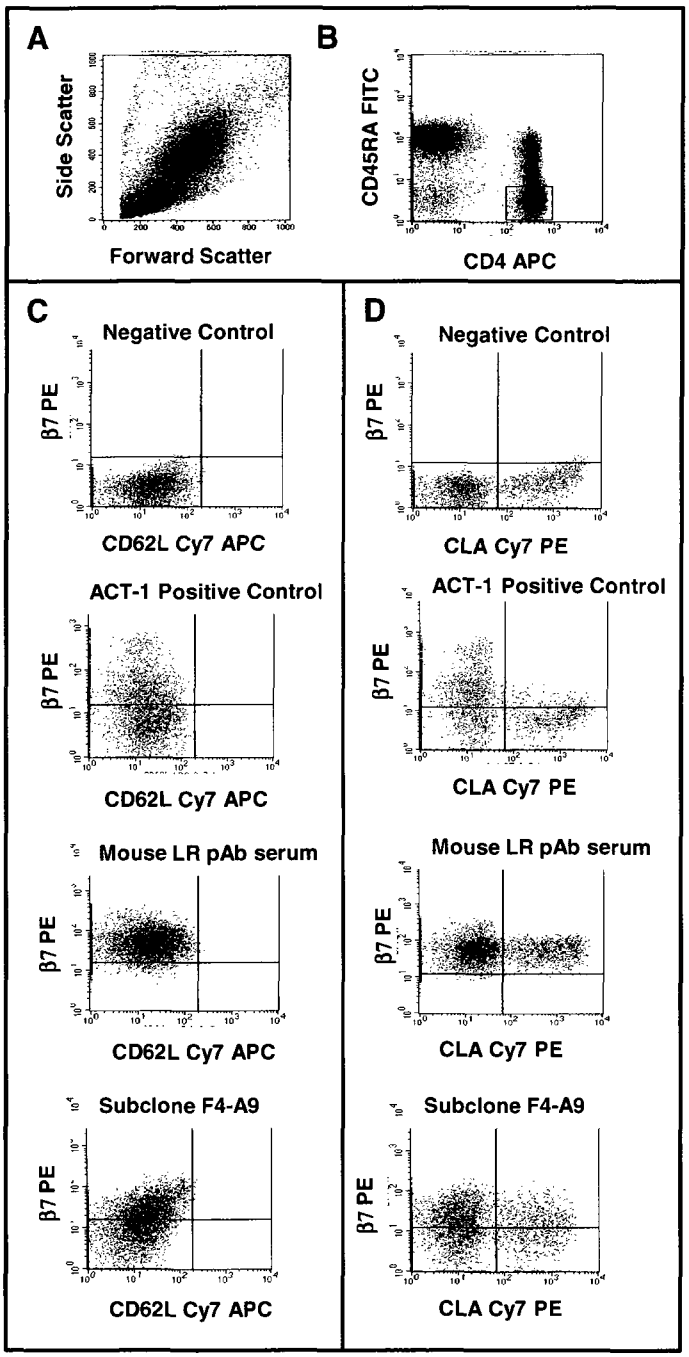

FIGURE 8. Multi-color flow cytometric screening of pAb serum and hybridoma subclone supernatants on effector memory T cells that migrate to the gut. A. The dot plot shows the region for a lymphocyte population, R1. B. The dot plot shows the region for CD4+ CD45RA ^{low} memory T cells, R2 . C. and D. Dot plots are gated on R1 and R2. PE is conjugated to goat anti-mouse secondary Abs (FL2-H). In C. Cy7 APC is conjugated to anti-CD62L Abs. In D. Cy7 PE is conjugated to streptavidin which binds biotinylated CLA.

Discussion

Along with a dysregulated mucosal immune system, IBD involves constant recruitment of lymphocytes circulating in the blood to the gut. The α4β7 integrin expressed by lymphocytes mediates their binding to the vascular endothelium via the MAdCAM-1 ligand and facilitates their transmigration into the gut tissue (2). In this study, an attempt was made to produce an anti-α4β7 Ab that could potentially be used to track and study gut-homing lymphocytes associated with IBD, since the only anti-α4β7 Ab in existence is not commercially available for research. β7 K/O mice were immunized with naturally-occurring β7 found on WT mouse splenocytes and PBMC. The rationale for immunizing with both mouse and human cells was to generate polyclonal Abs against shared epitopes of mouse and human β7. After an immune response was confirmed against the β7, hybridoma technology was used to generate clones secreting anti-β7 Abs. Screening assays were optimized to identify and isolate these hybridomas. The Ab produced, F4-A9, generated different staining patterns than the positive control, ACT-1 when binding to a subset of T cells that migrate to the gut. This indicates that the Ab is different from the ACT-1 mAb and unlikely to be anti-α4β7.

Our results indicate that the immunization strategy was effective in eliciting an immune response but could be improved. The naturally-occurring cell-surface β7 subunit on WT mouse splenocytes was immunogenic and induced an immune response in β7 K/O mice as evident by the β7 antigen-specific polyclonal Abs. The post-immune sera reacted with mouse β7 on TK1 cells and WT splenocytes as well as with human

β7 on PBMC but not with β7 K/O splenocytes. Non-specific binding of the sera to β7 K/O splenocytes may have been due to Abs generated against other epitopes on WT splenocytes, although ideally the only difference between the β7 WT mice and the K/O mice should have been the β7 molecule found on WT mouse cells.

The immune response was good but the number of plasma cells secreting anti-β7 Abs could be enhanced through several avenues such as increasing the frequency and number of immunizations or exploring other modes of antigen administration (19). In addition, alternative immunogens can be used such as synthetic β7 peptides conjugated to carrier proteins or T lymphoma cells over-expressing α4β7 (TK1).

The success of the next step in β7 specific mAb production, the fusion, relied on the successful union of myeloma cells with highly-specific plasma cells. Many factors regarding the two cells types must be accounted for in order to generate the desired number of hybridomas. An average of 265 hybridomas per fusion were obtained. Although a reasonable number, it could be increased with an improved technique. Essentially, the best way to get a better yield of anti-β7 Ab producing hybridomas would have been to have the optimal number of β7 specific plasma cells, rather than naïve B cells, available at the time of fusion. Furthermore, the ratio of the two cell types during the fusion is also an important factor in generating hybridomas (20). We used a 1:3 ratio of Sp2/O myeloma cells to splenocytes, although the ideal is 1:2. This may have affected our fusion, since there were many more splenocytes than their myeloma partners. Yet other factors influencing the fusion are the handler's technical experience and conditions

during the fusion. Gentle handling combined with the optimum temperature and media will ensure the survival of cells through stressful conditions.

Once the hybridomas were screened for β7 specificity, staining patterns of PBMC and TK1 cells were evaluated. Hybridoma supernatants stained the cells differentially. In some cases, for example hybridoma G5-H5, two distinct populations, one positive and the other negative for β7, were observed. More than one Ab might be present in the supernatants from these hybridomas, requiring subcloning. Additionally, since the cells may not all be in identical stages of the cell cycle, a dividing population may exhibit differential expression of cell-surface antigens. In some other cases, a range was observed in PBMC and TK1 cell staining. This can be explained as cells binding the Abs with different intensities due to the epitopes for Ab binding not being exposed the same on all cells.

When the supernatants were examined on β7 WT versus β7 K/O splenocytes, overall low fluorescence values and no reduction in binding to K/O cells was observed. This could be attributed to low Ab concentration, or it could mean the hybridomas lost the ability to produce Ab. Moreover, the pAb serum did not function as well as it did in previous experiments. Freeze-thaw cycles may have affected the integrity of the polyclonal Abs.

Functional analyses demonstrated that the Abs could not inhibit positive control Abs from binding β7. This could mean that these Abs bound different epitopes than the positive control Abs and therefore could not block. It could also mean that the Abs were

not blocking Abs, or it is possible that the supernatants again had low concentrations of Ab or no Ab at all.

Finally, the Abs were tested for binding to effector memory T cells known to migrate to the gut, and staining patterns were compared to those of the well-established ACT-1 mAb. Effector memory T cells that migrate to the gut have $\alpha4\beta7$, and those that migrate to the skin have CLA. Effector memory T cells stained with ACT-1 showed three populations, effector memory T cells that have CLA and migrate to the skin, those that do not have CLA but rather $\alpha4\beta7$ and migrate to the gut, and some that have neither. In the polyclonal Ab serum and the mAb samples, however, only two populations were observed: one that seemed positive for $\beta7$ but not CLA and another that seemed positive for both. The latter population is unusual because effector memory cells would express one type of homing molecule and suppress the other, resulting in migration to the gut or skin, not both. This indicates that the Abs in the polyclonal Ab serum as well as the mAbs are different from ACT-1 and unlikely to be anti-$\alpha4\beta7$.

In summary, this paper describes a novel immunization strategy to generate an $\alpha4\beta7$ mAb and the development of screening procedures to isolate specific anti-$\beta7$ secreting hybridomas. Further work will be needed to identify additional $\beta7$ specific hybridomas and stabilize mAb production in culture. Methods to optimize the immune response need to be evaluated. A better immune response, composed of Abs with improved $\beta7$ specificity, will make improved identification and isolation of hybridomas producing highly-reactive Abs with the desired specificity for $\beta7$.

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CHAPTER III

DISCUSSION

DISCUSSION

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Our results indicate that the immunization strategy was effective in eliciting an immune response but could be improved. The naturally-occurring cell-surface $\beta7$ subunit on WT mouse splenocytes was immunogenic and induced an immune response in $\beta7$ K/O mice as evident by the $\beta7$ antigen-specific polyclonal Abs. The post-immune sera reacted with mouse $\beta7$ on TK1 cells and WT splenocytes as well as with human $\beta7$ on PBMC but not with $\beta7$ K/O splenocytes. Non-specific binding of the sera to

β7 K/O splenocytes may have been due to Abs generated against other epitopes on WT splenocytes, although ideally the only difference between the β7 WT mice and the K/O mice should have been the β7 molecule found on WT mouse cells.

The immune response was good but the number of plasma cells secreting anti-β7 Abs could be enhanced through several avenues. First, the mice can be exposed to more of the antigen by increasing the number of splenocytes used for the immunizations or increasing the frequency and number of immunizations. Second, other modes of antigen administration can be explored, such as injecting footpads associated with lymph nodes rather than the s.c. or i.p. methods commonly targeting the spleen. Finally, alternative immunogens can be used such as synthetic β7 peptides conjugated to carrier proteins. Abs generated by synthetic peptide antigens have the advantage of binding denatured proteins. However, unlike whole cell generated Abs they may not bind globular proteins and have limited function (29). In contrast, Abs generated to whole cells may have a broader range of functional activities. The Abs can be fluorescently-tagged and used to stain cells expressing the epitope of interest and/or they can be used to block receptor/ligand interactions. If immunizations with whole cell antigens are continued for this study, immunizations with T lymphoma cells over-expressing α4β7 (TK1) can be used as an alternative. However, the cells would need to be treated with mitomycin C to repress cell growth prior to the immunizations.

The success of the next step in β7 specific mAb production, the fusion, relied on the successful union of myeloma cells with highly-specific plasma cells. Many factors regarding the two cells types must be accounted for in order to generate the desired

number of hybridomas. We obtained an average of 265 hybridomas per fusion. Although a reasonable number, it could be increased with an improved technique. Essentially, the best way to get a better yield of anti-β7 Ab producing hybridomas would have been to have the optimal number of β7 specific plasma cells, rather than naïve B cells, available at the time of fusion. More sources of plasma cells such as lymph nodes could have been sought along with the spleen. β7 specific plasma cells could also have been enriched by passing the splenocytes through column chromatography, with β7 molecules attached to column beads. In addition, β7 specific plasma cells could be sorted using fluorescently-tagged β7. Furthermore, the ratio of the two cell types during the fusion is also an important factor in generating hybridomas (30). We used a 1:3 ratio of Sp2/O myeloma cells to splenocytes, although the ideal is 1:2. This may have affected our fusion, since there were many more splenocytes than their myeloma partners. Moreover, it was once believed the histocompatible relatedness of the two types of cells being fused plays a role on hybridoma stability (30). Our fusion also involved cells from two different strains of mice; C57BL/6 splenocytes were fused with Sp2/O myeloma cells originating from a BALB/c mouse. The average of 265 hybridomas per fusion may have been higher if cells from the same strains had been used for the fusion. Yet other factors influencing the fusion are the handler's technical experience and conditions during the fusion. Gentle handling combined with the optimum temperature and media will ensure the survival of cells through stressful conditions. Hybridoma yield could have also been influenced by our plating method since we tried to obtain the maximum number of single clones by plating different concentrations of the fused cells. Although we were able to

get high percentages of single clones, this perhaps compromised our overall yield of positive hybridomas.

Screening assays need to be optimized for the identification and isolation of anti-β7 Ab-producing hybridoma cell lines. These assays must be able to process hundreds of samples simultaneously and identify positive clones within 48 hours (29). They must also be proven to be specific and sensitive for the antigen-Ab reaction. The ELISA, using TK1 cells, was able to process multiple samples; however, it was not as sensitive as the flow cytometry assays. Furthermore, unlike flow cytometry, in the ELISA, PBMC could not be mixed with TK1 cells to provide information simultaneously on both anti-human and anti-mouse β7 Abs. Although flow cytometry has these advantages, a disadvantage of flow cytometry is that samples can be prepared only in FACS tubes. This limits the number of samples that can be analyzed. Overall, our screening assays were reliable and reproducible; however, we need an assay that will combine efficiency with sensitivity.

Once the hybridomas were screened for β7 specificity, staining patterns of PBMC and TK1 cells were evaluated. Hybridoma supernatants stained the cells differentially. In some cases, for example hybridoma G5-H5, two distinct populations, one positive and the other negative for β7, were observed. More than one Ab might be present in the supernatants from these hybridomas, requiring subcloning. Additionally, since the cells may not all be in identical stages of the cell cycle, a dividing population may exhibit differential expression of cell-surface antigens. In some other cases, a range was observed in PBMC and TK1 cell staining. This can be explained as cells binding the Abs

with different intensities due to the epitopes for Ab binding not being exposed the same on all cells.

When the supernatants were examined on β7 WT versus β7 K/O splenocytes, overall low fluorescence values and no reduction in binding to K/O cells was observed. This could be attributed to low Ab concentration, or it could mean the hybridomas lost the ability to produce Ab. Moreover, the pAb serum did not function as well as it did in previous experiments. Freeze-thaw cycles may have affected the integrity of the polyclonal Abs.

Functional analyses demonstrated that the Abs could not inhibit positive control Abs from binding β7. This could mean that these Abs bound different epitopes than the positive control Abs and therefore could not block. It could also mean that the Abs were not blocking Abs, or it is possible that the supernatants again had low concentrations of Ab or no Ab at all. The hypothesis that the hybridomas lost the ability to produce Abs needs to be further examined.

Some clones may have lost the ability to produce Ab as a result of being in culture for an extended period of time. The stability of the clones in terms of Ab production may have been compromised due to high cell density (30). High cell density can lead to buildup of toxic waste material and competition for nutrients, and eventually cells may begin to self-regulate their growth and enter a lag phase. Some hybridomas may stop producing Ab, once they have experienced this event. In addition, studies have shown that cell proliferation and Ab production are not necessarily correlated (31). Even though some clones looked healthy and grew well, conditions for Ab production might not have

been met. Ideal serum concentrations, for example, have been shown to be different for cell growth versus Ab production. FCS was reduced to 10% from the original 20% in order to wean the cells from serum dependency. This sudden change could have led to a drop in Ig secretion. A more gradual change would have been preferred to allow the cells to adapt.

Loss of function of Ab-producing hybridomas can also be attributed to the unstable nature of the hybrid cell chromosome. Although it might not be true for our allogeneic fusion, it has been shown that in xenogeneic fusions the non-mouse myeloma genes compete with the mouse heavy chain immunoglobulin locus on the B cell chromosome for expression (30). In fact, the myeloma chromosome may even repress the B cell chromosome from translating Abs and go as far as to prevent replication of Ab genes. These explanations provide insight into our hybridomas' lack of ability to produce Ab. The next time we handle antibody-secreting hybridomas, we would ensure the clones are screened while they are in log phase of growth before they enter a lag phase and stop producing Ab.

Finally, the Abs were tested for binding to effector memory T cells known to migrate to the gut, and staining patterns were compared to those of the well-established ACT-1. Effector memory T cells that migrate to the gut have $\alpha 4\beta 7$, and those that migrate to the skin have CLA. Effector memory T cells stained with ACT-1 showed three populations, effector memory T cells that have CLA and migrate to the skin, those that do not have CLA but rather $\alpha 4\beta 7$ and migrate to the gut, and some that have neither. In the polyclonal Ab serum and the mAb samples, however, only two populations were

observed: one that seemed positive for β7 but not CLA and another that seemed positive for both. The latter population is unusual because effector memory cells would express one type of homing molecule and suppress the other, resulting in migration to the gut or skin, not both. This indicates that the Abs in the polyclonal Ab serum as well as the mAbs are different from ACT-1 and unlikely to be anti-a4b7.

Once an anti-β7 Ab is identified from the hybridomas, it can be characterized and its function further analyzed. Like the competition assays where the Abs were tested for the ability to block positive controls from binding β7, a chemotaxis assay can prove useful in assessing Ab function. This assay can test if the anti-α4β7 Ab is able to inhibit gut-specific cells expressing α4β7 from migrating to the α4β7 ligand, MAdCAM-1, thus interfering with interaction essential for gut-homing.

An anti-α4β7 Ab may be used to test the hypothesis that IBD is associated with elevated blood levels of gut-homing plasmablasts. Plasmablasts are premature plasma cells that express α4β7 if they are destined to migrate to the gut. This Ab would be able to track and quantify gut-specific plasmablast levels in patient blood samples and monitor how the levels change in response to commonly-used therapies against IBD. This would be accomplished by FACS analysis of PBMCs from IBD patients using plasmablast markers such as CD19+, CD38+, CCR10 and CCR9 along with the α4β7 integrin. CD19 and CD38 are markers found on plasma cells, while CCR10 and CCR9 are involved in cell trafficking and migration to sites of inflammation. Additionally, it is important to observe plasmablasts in the blood, because several cases of IBD treated with anti-TNFα mAb have resulted in rare and fatal plasmablast lymphomas (32). Studying

plasmablasts may shed light on more aspects of the commonly used anti-TNFα mAb treatment. An anti-α4β7 Ab would provide the means to look into these clinical and research issues.

In summary, this paper describes a novel immunization strategy to generate an α4β7 mAb and the development of screening procedures to isolate specific anti-β7 Ab-secreting hybridomas. Further work will be needed to identify additional β7 specific hybridomas and stabilize mAb production in culture. Methods to optimize the immune response need to be evaluated. A better immune response, composed of Abs with improved β7 specificity, will make improved identification and isolation of hybridomas producing highly-reactive Abs with the desired specificity for β7.

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APPENDICES

APPENDIX A. IACUC APPROVAL LETTER

San Jose State University
Institutional Animal Care and Use Committee

LETTER OF OFFICIAL PROTOCOL REVIEW

Date: July 24, 2007

Dear Dr Abramson,

The animal care and use portion of your research proposal indicated below was reviewed by the Institutional Animal Care and Use Committee (IACUC). The status of your proposal is as follows:

Principal Investigator/s: Tzvia Abramson
Co-investigator/s: Asima Khan
Protocol #: 904
Title: Bio-233 Lab Unit: Production of Specific Immune Response in Balb/c mice for production of Monoclonal Antibodies.

The application was approved without modification by the IACUC.

Approval date: August 22, 2007 * Expiration Date: August 21, 2010

The IACUC must be informed in writing of any proposed changes to the approved protocol outline and approval must be granted in writing by the IACUC before any change is instituted. If you wish to continue the approved outline beyond the expiration date, it is required that you resubmit an animal care and use application for IACUC review and approval in June 2010.

The protocol number (#904) may only be used by the instructor and participants included on the approved application form. The protocol number will be required to order animals for this study (maximum 105 mice). Submitting animal order and delivery requisitions is the responsibility of the Principal Investigator and orders are to be placed through the University Animal Care office in ample time before the study. If you have any questions, feel free to contact me at extension 924-4929.

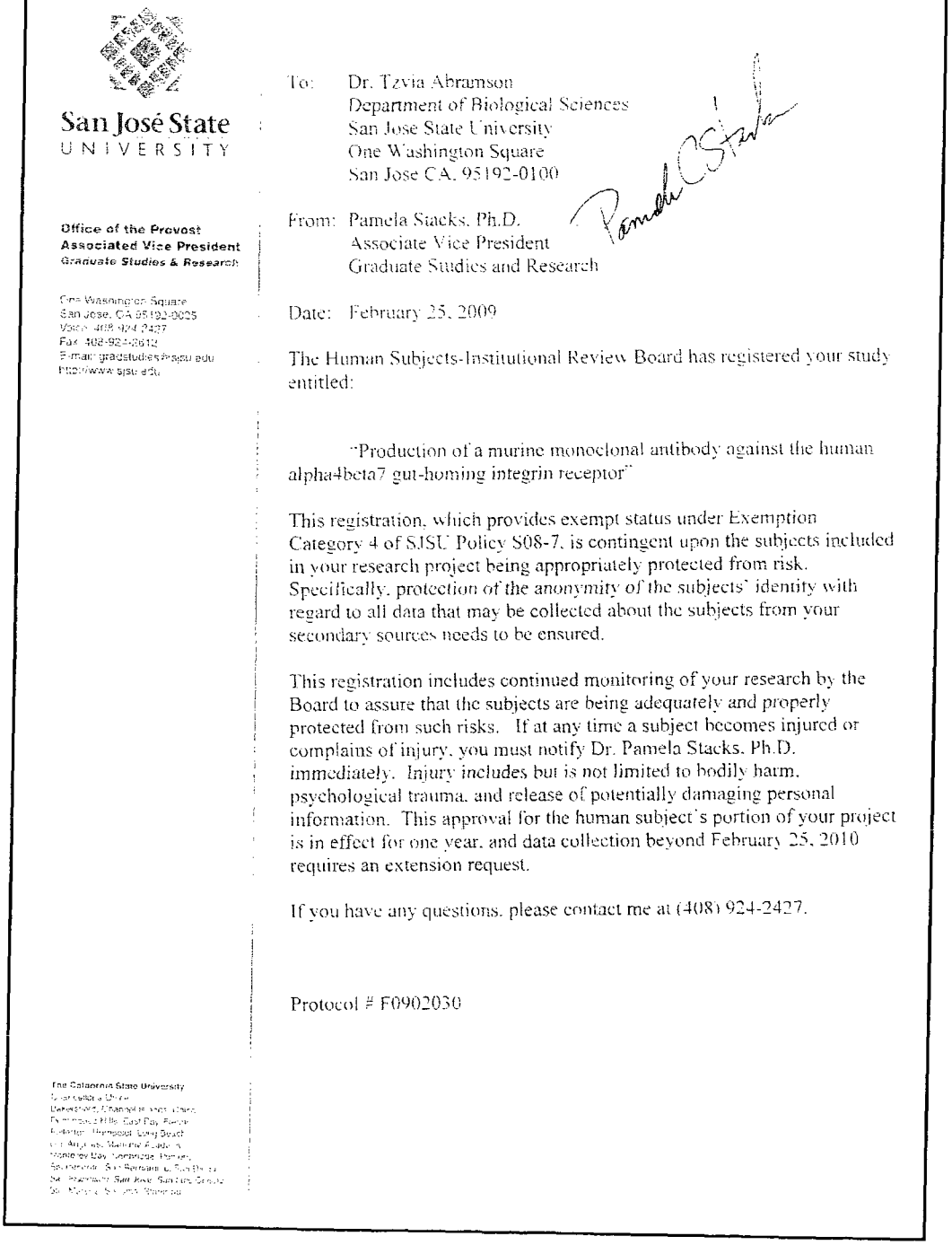
Larry Young, RVT, CPIA
IACUC Coordinator

Cc: IAC Office

This protocol has been approved as a Health Risk Category Two level project (RC-2).

Please refer to the attached risk category description page for relevant personnel safety information pertaining to this study.

APPENDIX B. IRB APPROVAL LETTER



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To: Dr. Tzvia Abramson
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From: Pamela Stacks, Ph.D.
Associate Vice President
Graduate Studies and Research

Date: February 25, 2009

The Human Subjects-Institutional Review Board has registered your study entitled:

"Production of a murine monoclonal antibody against the human alpha4beta7 gut-homing integrin receptor"

This registration, which provides exempt status under Exemption Category 4 of SJSU Policy S08-7, is contingent upon the subjects included in your research project being appropriately protected from risk. Specifically, protection of the anonymity of the subjects' identity with regard to all data that may be collected about the subjects from your secondary sources needs to be ensured.

This registration includes continued monitoring of your research by the Board to assure that the subjects are being adequately and properly protected from such risks. If at any time a subject becomes injured or complains of injury, you must notify Dr. Pamela Stacks, Ph.D. immediately. Injury includes but is not limited to bodily harm, psychological trauma, and release of potentially damaging personal information. This approval for the human subject's portion of your project is in effect for one year, and data collection beyond February 25, 2010 requires an extension request.

If you have any questions, please contact me at (408) 924-2427.

Protocol # F0902030

The California State University
Bakersfield, Channel Islands, Chico,
Dominguez Hills, East Bay, Fresno,
Fullerton, Humboldt, Long Beach,
Los Angeles, Maritime Academy,
Monterey Bay, Northridge, Pomona,
Sacramento, San Bernardino, San Diego,
San Francisco, San Jose, San Luis Obispo,
San Marcos, Sonoma, Stanislaus