

AN ABSTRACT OF THE THESIS OF

Drew W. Calhoun for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on June 8, 2007. Title: Generation of Proof of Concept Molecules: Neutralizing Monoclonal Antibodies to IL17Cm.

Abstract approved: _____

Kevin Ahern

Inflammation is one of the first, most important responses of the immune system when presented with infection and is mediated primarily by eicosanoids and cytokines. The goal of this research was to generate monoclonal antibodies against murine IL17C, a poorly characterized cytokine involved in inflammation and linked to inflammatory conditions. The antibodies generated were then screened for their ability to block the interaction between IL17Cm and its receptor, IL17RE, or to bind IL17Cm without blocking this interaction. Three sets of mice were immunized with IL17Cm and various immunomodulators and screened for antibody titer development. The mice numbered 18447-18550 were sacrificed and their secondary lymphoid organs were used for a hybridoma fusion. The supernatants of the viable, antibody-producing hybridomas were screened for neutralization and the cells were frozen. There were 12 samples from the fusion (#346) that were determined to produce anti-IL17Cm antibodies. Supernatant samples 1 and 9 showed potential neutralizing ability while 1, 11, 21, and 36 demonstrated activity characteristic of non-blocking antibodies. Purification of the supernatants by cloning and further confirmation screening must be done to validate these results; however, the antibodies produced look promising for future proof of concept studies and as assay reagents for further research into IL17C and its receptor.

Key Words: IL17C, inflammation, cytokines, monoclonal, antibody

Corresponding e-mail address: DrewCalhoun3@gmail.com

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Generation of Proof of Concept Molecules
Neutralizing Monoclonal Antibodies to IL17Cm

by

Drew W. Calhoun

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Biophysics (Honors Scholar)

Presented June 8, 2007
Commencement June 17, 2007

Honors Baccalaureate of Science in Biochemistry and Biophysics project of Drew W. Calhoun presented on June 8, 2007.

APPROVED:

Mentor, representing Biochemistry and Biophysics

Committee Member, representing Biology

Committee Member, representing ZymoGenetics

Chair, Department of Biochemistry and Biophysics

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Drew W. Calhoun, Author

Acknowledgements

First, I'd like to thank all of the wonderful people at ZymoGenetics for the amazing opportunity they provided for this research. I'd especially like to thank Mark Rixon and Ursula Garrigues, as well as the rest of the monoclonal antibody group of the Protein Biochemistry of ZymoGenetics. Johanna Harshman deserves a special thank you for the tremendous amount of work she put in to assist me in the majority of all the research I did as well as teach me about the inner workings of ZymoGenetics.

My mentors, advisors, professors, and peers at Oregon State University are also deserving of all my thanks. I'd especially like to thank my main advisors, Dr. Kevin Ahern and Dr. Indira Rajagopal, who have provided constant support and advice not limited to my thesis, but for the entire duration of my tenure here in Corvallis. I look forward to a life-long personal and professional relationship with these wonderful people. The University Honors College experience as a whole has been a wonderful experience and I'd like to thank all of the people who make it happen for their tremendous work and for even instilling the thought in my head of completing this thesis work, it has been an incredibly rewarding experience.

Finally, I'd like to thank my family and friends for their constant support, advice, and love. Of all these people, I'd especially like to thank my mother, Jill Calhoun, for her unconditional love and support in all arenas of my life. Without her, even besides her biological contributions nearly 22 years ago, none of this would be possible and for that, I am eternally grateful. Thank you mom!

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To all of you...
...except for Gautam Naresh Mankaney

Generation of Proof of Concept Molecules: Neutralizing Monoclonal Antibodies to IL17Cm

Introduction

Immune System and Response Overview

Put simply, an organism's immune system is the collection of cells and molecules that are responsible for protection from disease. The coordinated response which the immune system generates to identify and eliminate the pathogens that lead to such infection is known as the immune response, and it involves a complex array of layered defenses (with increasing specificity) from such infection [1]. The immune response begins with the immediate and rapid, yet non-specific, reactions of the innate immune system. Besides the apparent physical barriers (e.g. skin), the innate arm of the immune system involves phagocytic and natural killer cells and a variety of proteins including 'complement' (a system of proteins involved in immune responses) and various mediators of inflammation, one of the first responses of the immune system [1]. While the innate arm of immunity allows for an immediate response to infection without the need for prior immunization, it lacks specificity. This is where the adaptive arm of the immune response comes into play. The adaptive response is stimulated by exposure to the pathogen and develops as a result of it. While the adaptive arm is slower in response time, it makes up for it in specificity, diversity, and memory. In essence, exposure to the pathogen serves as a later signal for the adaptive immune system, priming it to generate, and "remember" for subsequent exposures, a specific response adapted especially for the antigen presented [1]. The

adaptive immune response is carried out using lymphocytes, specifically bone marrow-derived lymphocytes (B-cells), which are the precursors of antibody-forming cells, and thymus-derived lymphocytes, which regulate the action of other cells (T-cells) [2]. For normal and appropriate physiological functioning, both the innate and adaptive immune responses have, and depend on, the ability to distinguish between self and non-self molecules [2]. Hence, it is of primary importance to note that when this ability is compromised, serious problems can arise.

Inflammation

Inflammation is one of the first and most important responses of the immune system when presented with infection. It can literally be defined as “the body’s way of dealing with infections and tissue damage” [3]. Inflammation is mediated primarily by eicosanoids and cytokines. The increased blood flow to a tissue associated with inflammation leads to the redness and swelling that characterize its externally observable symptoms. Inflammation must be closely regulated however, because there is a “fine balance between the beneficial effects of inflammation cascades and their potential for long-term tissue destruction [3].” Indeed, overactive immune responses can be as detrimental as immunodeficiencies. In fact, the development of certain diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis may result from inflammation cascades when that fine balance is tipped [3]. Of primary importance to the regulation of the inflammatory response, as well as to the immune response as a whole, is the activity of cytokines, which play a central role in the mediation of cellular and physiological immune responses [4].

Cytokines

Cytokines are soluble proteins that are secreted by leukocytes and other cell types. They act as chemical communicators between cells to regulate many biological activities related to immune responses [3]. Cytokines constitute a large family of proteins with about 93 identified members and about 96 identified receptors, many with functions yet to be elucidated [3]. Cytokines were originally termed ‘factors’ based on their biological activities (e.g. tumor necrosis factor). However, this has changed with the recognition of their abundant pleiotropic and redundant activities with expanded knowledge due to recombinant cloning and the current era of genomics [5]. Indeed, cytokine involvement is apparent in almost every aspect of immunity and inflammation, ranging from induction of the innate immune response to the generation of cytotoxic T-cells and antibody-development by the humoral arm of the adaptive response [5]. In regards to haematopoiesis, cytokines have been shown to regulate the survival and proliferation of haematopoietic progenitor cells, as well as the induction of their lineage-specific differentiation [6]. Many cytokines are also proinflammatory and contribute, either directly or indirectly, to the development of inflammation and, at times, can lead to chronic autoimmune disease pathologies [3]. Due to the pleiotropic roles of cytokines, it is the actual combination of cytokines produced in response to an “immune insult” that effectively determines which arm of the immune system will be activated [5].

While the term ‘cytokines’ refers to a large family of proteins, there are many structurally and functionally distinct subfamilies of proteins and genes within it, of which the primary focus here will be those families made up of interleukins. Interleukins are cytokines primarily produced by T-cells, which themselves are inextricably involved in

the initiation and control of immune responses [3]. Such T-cell-derived cytokines are thought to be pivotally important in inflammation and many serve as potential targets for drug therapies. However, before drug therapies can be developed, cytokine functions must be elucidated and this requires an understanding of their secretion, the receptors they associate with, and the signal transduction pathways they induce [7].

The process of cytokine involvement in immune responses begins with their production as a local reaction at any site of injury or infection [8]. In particular, cytokines derived from mononuclear phagocytic cells and other antigen-presenting cells are extremely effective in promoting inflammation. The production of cytokines may be achieved either by monocytes, which are potently triggered to produce them through the innate immune system using pattern recognition receptors to recognize the pathogen, or by the activity of the antigen-presenting cells (APCs). The APCs promote the adaptive arm of immunity by taking up the antigen, metabolizing it, and presenting it to the T helper lymphocytes, which results in the release of cytokines, this being part of the cell-mediated aspect of the adaptive arm. These cytokines, produced by the T-helper (Th) lymphocytes, are critical for the regulation of both protective and pathogenic immune responses [9].

The combination of cytokines that are released by T-helper lymphocytes depend on the type of T helper cell type that they are derived from. In other words, the process in which naïve T cells differentiate into effector T cells with enhanced functional potential is initially under the primary guidance of cytokines produced by pathogen-activated cells of the innate immune system [10]. This differentiation into effector T cells is driven by the antigen that was originally presented and as a result, it may lead to different subsets

of effector T cells being produced depending on the cytokines activated. Originally, there were only two classes of these CD4 T cells identified, the T-helper type I lymphocytes (Th1) and the T-helper type II lymphocytes (Th2). Both of these effector cell types were derived from naïve T cells and are defined by the cytokine receptors that were activated upon the initiation of the immune response; basically, Th1 cells were primarily induced by the activation of Type I receptors and Th2 cells by Type II receptors. These different subsets of T cells produce distinct profiles of cytokine secretion which induce each of their reciprocal patterns of immunity, that being cell-mediated immunity in Th1 cells and humoral immunity in Th2 cells [11]. While initial cytokine signals induced by the innate responses resulted in the initial effector T cell development, it is interesting to note that some of the cytokines produced by these effector cells actually induce the differentiation of additional effectors of the same phenotype [12]. In this way, via their secreted cytokines, each subset promotes its own development while inhibiting the development of the other subset [13]. While this so-called “Th1-Th2 paradigm” helped establish an initial understanding of T cell development and the interplay between innate and adaptive immunity, a new effector T cell lineage, known as Th17, characterized by its involvement with the emerging IL17 family of cytokines, provides a new arm of adaptive immunity and further information into the cross-talk between the adaptive and innate immune responses [10]. It has also helped explain many previously confusing aspects of the immune system and continues to do so. For this reason, it is of particular interest to researchers.

The IL17 family and the associated Th17 subset is also of primary importance because of their proinflammatory characteristics, which have been linked to murine

models of host defense, as well as the less-desirable effects of chronic inflammatory disease including arthritis, colitis, encephalitis, myocarditis, and asthma/allergy [14]. Specifically, members of this family have been shown to activate, both directly and indirectly, the NF- κ B pathway, a classic inflammatory signaling pathway activated by diverse stimuli especially including inflammatory cytokines [15]. NF- κ B, specifically known as “nuclear factor- κ B, has been identified as a widely expressed and inducible transcription factor, which regulates the expression of many genes involved in mammalian immune and inflammatory responses [16].” While the protective functions of the NF- κ B signaling pathway cannot be ignored, the inflammation cascades it induces are often the root of the problem in many of the chronic inflammatory diseases.

Drug Development

Due to the fact that inflammation cascades often can lead to the development of various diseases, including rheumatoid arthritis and chronic asthma, blocking the output of inflammatory pathways is often the goal of many anti-inflammatory drug therapies. One way in which this can be done is through the antagonism or neutralization of a molecular target deemed pivotal in the inflammatory pathway [3]. Of course, many factors should be taken into consideration when looking for a well-suited drug target, in order to ensure sufficient efficacy, as well as minimal risk. For example, to ensure efficacy the potential drug target should be proximal to the initiation of the disease or, at least, play some sort of “pivotal role” in the disease process. Meanwhile, a drug target that is unique to the disease process would help reduce the risk involved because it could deliver the desired effect on the disease without detrimental and unwanted side effects on other processes [3].

For this reason, cytokines are often prime targets for drug therapies, especially considering that many are proinflammatory and directly contribute to the development of inflammation and chronic autoimmune disease pathologies [3]. Already, the utilization of cytokines has yielded a “rich stream of drugs” and this success, coupled with the growing number of cytokines and cytokines receptors continually being identified, supports the continued intensive focus on their potential as drug targets. In fact, all of the cytokines are potential molecular targets and could be exploited as therapeutics directly or

neutralized by various antagonist agents [3]. (These ‘potential targets’ can be seen in [Figure 1] to the right).

The present focus, however, seems to be on the use of the antagonistic or neutralizing agents against the actions of proinflammatory cytokines, due to the great successes

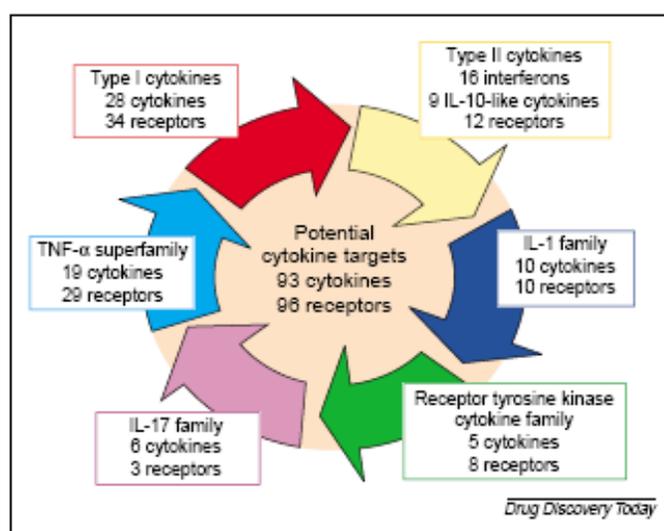


Figure 1: Potential Drug Targets - Cytokines

biopharmaceutical companies have had using monoclonal antibodies and receptor-Fc fusion proteins [3]. Unfortunately though, existing therapies that target common proinflammatory cytokines, such as TNF- α have a “high risk for infectious complications,” due to the extent of their immunity inhibition [10]. Likewise, recent strategies that utilized anti-p40 neutralizing antibodies have shown superb efficacy, but also high risk, due to the inhibition of both Th1 *and* Th17 immunity. Hence, there is good reason to believe that targeting more specific cytokines, such as individual members of

the IL17 family may be advantageous to establishing high efficacy with decreased risk of infectious complications [10].

Monoclonal Antibodies

As discussed above, the utilization of monoclonal antibodies as neutralizing agents to cytokine activity in drug therapies has proven extremely successful for various biopharmaceutical companies. Antibody production itself is a vital part of the immune response, and is a hallmark of adaptive immunity, primarily carried out by B lymphocytes to target invading pathogens for destruction [17]. Humans have millions of B cells, each of which carries around 50,000 antibody molecules on its surface, all being specific for a single epitope on an antigen [17]. Put simply, epitopes are small segments of antigens, recognized as non-self (foreign to the body), that serve as binding sites for antibodies and T-cell receptors. There can be few or several epitopes on a single antigen, but the general rule of thumb is that the greater the difference in sequence of the protein from “self,” the more antigenic it will be and hence, the greater the number of epitopes it will contain [2]. The abundance of B cells, coupled with the number of antibodies each has, allows the immune system to recognize as many as 10^8 epitopes at any given time [17].

This immense diversity is due, in part, to the antibody’s structure, specifically its antigen-binding site. An antibody has a symmetrical structure resembling the shape of the letter ‘Y’ and consists of two light chains and two heavy chains, which are held together by strong non-covalent forces and disulfide bonds. There are five classes of antibodies that are distinguishable by their heavy chains and named according to them (e.g. the IgG

antibody contains γ heavy chains). The antigen-binding site, or F_{ab} region, is comprised of six short loops (three from each chain) and these facilitate tight, specific, and reversible epitope binding. The F_{ab} region of the antibody is also known as the hypervariable region, and it actually contains the complementarity-determining regions (CDRs), which actually bind the specific epitope [17]. The heavy chains alone continue down into the constant region at their C-terminal end. This is called the F_c (crystallizable) region. Upon binding of the antigen, the F_c region's role is to activate 'effectors,' such as the complement system, or to trigger the phagocytosis process to eliminate the given pathogen. A schematic of the antibody's structure is shown here in [Figure 2].

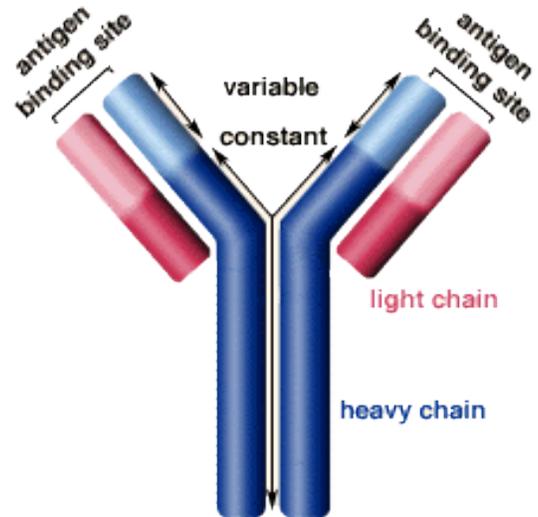


Figure 2: Antibody Schematic

The clonal selection theory suggests that antibody binding triggers the clonal expansion of B cells either into plasma cells, committed to making only one specific antibody after stimulation, or memory B cells, which prime immune responses for future exposure [17]. The differentiation observed here is carried out by and in secondary lymphoid organs, such as the spleen and lymph nodes [2]. This differentiation also induces a very strong immune response with several different antibodies being produced, each with different specificities and affinities. Such a strong, polyclonal response enables the immune system to protect the body against a plethora of pathogens, but most often researchers aim to only recognize a single epitope on a single molecule (and for good reason) [17].

While a polyclonal response is beneficial *in vivo* for destroying pathogens, the same benefits are not seen in the administration of polyclonal drug therapies, such as a polyclonal antiserum. This is because such an antiserum lacks specificity and contains antibodies that target antigens on both diseased and healthy cells and proteins [18]. Indeed, an effective disease treatment depends on the use of a single antibody targeted to a specific epitope. As Markus Enzelberger, from the biotechnology company MorphoSys, explains, “epitope-specific monoclonal antibodies are necessary to detect subtle differences, such as splice variants or differences in protein folding.” Hence, in fields such as pharmaceuticals where antibody properties need to be consistent and well-characterized, a monoclonal antibody is essential [18]. In fact, monoclonal antibodies are exquisitely sensitive and enable researchers to explore complex cell structures and pathways extensively by the selective blocking of specific proteins in signaling or regulatory pathways [17].

The production of monoclonal antibodies was first developed in 1975 by Cesar Milstein and Georges Kohler as they developed a technique to construct a continuously growing cell line expressing a specific, predefined antibody, a discovery which was rewarded with a Nobel Prize nine years later [19]. The revolutionary technique combined the antibody-producing properties of B lymphocytes with the immortality and indefinite division seen in murine myeloma cells. This was actually done by the direct fusion of spleen cells with myelomas to produce antibody-producing hybridomas [17]. Researchers could then select individual hybridomas that produced specific, predefined monoclonal antibodies. This method has led to the development of various diagnostic antibodies and 18 monoclonal antibodies have already been approved for therapeutic use in the US [17].

While the use of antibody-producing hybridomas to generate monoclonal antibodies remains at the forefront, the protocol generally takes almost a month longer than polyclonal antibody development and there are various ways current methods could be improved to shorten this time [17]. For example, most methods for such monoclonal antibody generation require purified protein antigen on the microgram scale [20]. Recent technologies have devised a way for producing antibodies more easily, using genetic immunization of animals [21]. Advantages of such a technique include a high-throughput capability and high specificity, as well as the fact that antibodies produced from immunized animals are more likely to recognize the *native* protein [22]. Furthermore, linking the antigen gene to various elements known as immunogenic carriers, such as GM-CSF (granulocyte macrophage-colony stimulating factor), helps increase the immunogenicity of the protein in a non-specific manner and most often leads to an increased antibody titer [2, 23]. Continuously improving techniques, such as these, for developing hybridomas and monoclonal antibodies in a time-efficient manner must be considered and adapted as the importance and utilization of monoclonal antibodies continues to increase.

The vast number of drug therapies that are in the approval process as well as those that have already been approved provide significant hope for further therapies based on monoclonal antibodies. Indeed, this potential for drug utilization, as well as the extended studies that can be done on cytokines by using monoclonal antibodies, provides good reason for the development of such antibodies against members of the new, emerging IL17 cytokine family, and specifically for our purposes, IL17C.

IL17C

The IL17 family was first identified in 1995 with the discovery of its first member, IL17A, a protein with no known sequence similarity to any other cytokine or mammalian protein [5]. Since then, five other members (IL17B-F) have been identified and cloned. While the sequence similarity of these members with IL17A is limited (as low as 16% with IL17E and up to only 50% similarity with IL17F), members of this family share *no* sequence homology with other known mammalian proteins and therefore constitute a distinct cytokine family [10]. Family members have molecular weights ranging from 35 to 52 kDa and, at best, have overlapping, not identical, biological activities. All members, however, contain 4 strictly conserved cysteine residues which form a cysteine knot and all are also thought to have primarily proinflammatory properties [7, 10].

While certain members of the IL17 family, especially IL17A and IL17F, have been well-characterized and extensively researched, IL17C remains one of the least characterized. Murine IL17C is 40 kDa and has 26% homology with IL17A and 83% homology with the human equivalent [10]. It is also known to be expressed in a wide range of tissues, and has been detected in human testes, thymus, spleen, and the prostate [24]. While its proinflammatory functions are thought to be synergistic with those caused by IL17A, they are clearly distinct since IL17C doesn't induce IL6 secretion like IL17A, but does induce the release of TNF- α and IL-1 β from the monocytic cell line, THP-1 [25]. The cytokines TNF- α and IL-1 β have long been known to be proinflammatory, and possibly lead to autoimmune disease, but clinical strategies to block these cytokines have high risk for infection [26]. Hence, strategies to block IL17C may prove to be more promising in this sense.

According to most current sources, the receptor that binds to IL17C is largely unknown, as is the ligand that binds to the IL17RE receptor, although its expression has been seen in the human brain, prostate, and pancreas [5, 7, 10, 24, 27, 28]. The limited knowledge of IL17C has led it to be of primary interest to ZymoGenetics as a “potential novel opportunity.” Indeed, previous studies at ZymoGenetics have already shown that IL17C binds to IL17RE with high affinity and is able to induce the activation of the NF- κ B pathway in IL17-transfected cells. There is also evidence that IL17C has a role in inflammatory disease. For instance, it is up-regulated in a number of inflammatory conditions, both human and mouse. In humans, such up-regulation has been seen in inflamed skin, inflamed gut, and lung tissue from hypersensitivity pneumonitis, as well as the synovial fluid from patients with rheumatoid arthritis. In murine models, IL17C is highly expressed in inflamed paws from collagen-induced arthritis (CIA), gut tissues, and bronchoalveolar lavage (BAL) cells. The functional consequences have been demonstrated in various ways including the experimental augmentation of IL17C levels by retroviral infection, which proved to exacerbate collagen-induced arthritis.

Such strong evidence leads us to believe that IL17C plays an important role in maintaining these inflammatory conditions and that the IL17C/IL17RE interaction plays an important role in initiating and also maintaining disease conditions. If, indeed, the interaction between IL17C and IL17RE plays such a vital role, then the inhibition of their binding has significant potential for therapeutic utility. Hence, my ultimate research goal was to interrupt this interaction by generating monoclonal antibodies against murine IL17C. These antibodies will be useful for proof-of-concept studies to verify such hypotheses and also as useful assay reagents. For example, as an immunohistochemistry

reagent, the antibodies might be used to determine levels of IL17RE in normal and diseased tissues. I also conducted preliminary tests on the neutralizing ability of the monoclonal antibodies I developed. Specifically, the anti-muIL17C monoclonal antibodies generated were screened for their ability to block the binding and subsequent NF- κ B pathway activation of biotinylated IL17C in IL17RE-transfected cells. The neutralizing antibodies developed may be used to modulate disease progression in murine disease models and perhaps (much further down the line) may eventually lead to potential drug therapies.

Materials & Methods

Immunization

The production of anti-IL17Cm monoclonal antibodies began with the immunization of ten mice of the Balb/c strain, a strain of small, albino mice commonly used in research settings. The mice were ear-tagged and numbered from 18541-18550. Each mouse received an initial subcutaneous immunization on May 9th, 2006, which was administered to flank and neck regions. These were followed by three intra-peritoneal (IP) boosts, each delivering 100µg of protein, every two weeks, the last being on June 20th, 2006. The 1st and 2nd bleeds were taken one week after each of the last two IP boosts.

There were three immunization profiles that were used, each using an initial protein concentration of 1.9 mg/mL, which resulted in a 500µg/500µL end concentration per vial. The adjuvant used in each case was Titermax Gold. Mouse numbers 18541 and 18542 received the non-conjugated IL17Cm protein. Mouse numbers 18543-46 received IL17Cm conjugated to BSA, to increase immunogenicity. Finally, mice 18547-18550 received IL17Cm conjugated to BSA with CPG and GM-CSF additives, also used as immunomodulators.

Preliminary Screening

Following immunizations, the first and second bleeds were analyzed for antibody titer, the production of antibodies in response to the immunizations with the ability to bind the ligand, IL17Cm. The antibody titer developed by the ten mice was analyzed using Direct ELISA and Capture ELISA screening methods.

Direct ELISA (Enzyme-Linked Immunosorbent Assay)

Six 96-well plates (Nunc Maxisorp Certified #439454) were initially coated with “coating antigen” at 100 μ L/well and diluted in ELISA A buffer (0.1 M Sodium Carbonate at pH 9.6). Two plates were the experimental setups, coated with IL17Cm at a 1 μ g/mL concentration. The other four plates were control setups, two coated with an unrelated protein, IL27, and two others simply containing BSA diluted 1% (weight/volume). The six coated plates were sealed and incubated overnight at 4 C°. The plates were then washed two times with 300 μ L per well of ELISA C (PBS with 0.1% Tween-20). Plates were then blocked with 200 μ L per well of BSA/Tween and incubated for 1 hour at room temperature. Plates were emptied by flicking and then loaded with 50 μ L per well of antibody samples according to the plate map shown on the following page. *Note: The second plate in each series consisted of mouse numbers 18546-18550.* After antibody samples were loaded, the plates were incubated for another hour at room temperature before two more ELISA C washes. The plates were once again emptied and the secondary antibody, diluted in the ELISA B buffer (ELISA C with 1% BSA), was added. The secondary antibody, an Fc-specific goat anti-mouse IgG linked to SA-HRP (Streptavidin conjugated to horseradish peroxidase), was diluted 1:5000 in the ELISA B and 100 μ L per well was added to all plates before another 1 hour incubation at room temperature. Plates were finally washed five more times with ELISA C, flicked to empty, and plated with 100 μ L/well of TMB development solution. After 3 minutes of room temperature incubation, the color development was stopped with 100 μ L/well of Stop solution. Absorbance was read at 450nm within 15 minutes of the STOP addition with the SoftMax Pro program and associated plate reader.

Table 1.1: Plate 1 Antibody Dilution Map

Mouse # - Bleed #					
18541-1	18542-1	18543-1	18544-1	18545-1	NMS
1:1000	1:1000	1:1000	1:1000	1:1000	1:1000
1:10000	1:10000	1:10000	1:10000	1:10000	1:10000
1:100000	1:100000	1:100000	1:100000	1:100000	1:100000
1:1000000	1:1000000	1:1000000	1:1000000	1:1000000	1:1000000
1:1000	1:1000	1:1000	1:1000	1:1000	1:1000
1:10000	1:10000	1:10000	1:10000	1:10000	1:10000
1:100000	1:100000	1:100000	1:100000	1:100000	1:100000
1:1000000	1:1000000	1:1000000	1:1000000	1:1000000	1:1000000
18541-2	18542-2	18543-2	18544-2	18545-2	NMS

Capture ELISA (Enzyme-Linked Immunosorbent Assay)

The Capture ELISA method is very similar to the Direct ELISA and simply adds the antibodies and protein in a different order, and the protein is also biotinylated as part of this method. For the Capture ELISA screening at this stage, only two plates were used and the addition of proteins on the plate was modified accordingly to maintain analysis of the two control setups. The two plates were initially coated with 100 μ L/well of the “coating antibody,” a goat anti-mouse Fc-specific IgG, which was diluted to 1 μ g/mL in ELISA A. After coating, the plates were sealed and incubated at 4 C° overnight. The plates were then washed two times with 250 μ L/well of ELISA C, flicked to empty, blocked with 200 μ L/well of 1% BSA/Tween, and incubated 1 hour at room temperature. The antibody samples were then plated, 50 μ L/well, and according to the same plate map used previously. After the antibody samples were loaded, plates were incubated for

another hour at room temperature and then washed two times with 250 μ L/well of ELISA C. The specific biotinylated proteins (biotinylation process described below) were then diluted in ELISA B buffer to a 0.5 μ g/mL final protein concentration and plated 100 μ L/well. Plates were then incubated for 1 hour at room temperature in the dark. Following incubation, plates were washed two times with 250 μ L/well of ELISA C buffer and SA-HRP (diluted 1:2000 in ELISA C) was added 50 μ L/well and plates were once again incubated in the dark for 1 hour at room temperature. Plates were finally washed five times with 250 μ L/well in ELISA C, plated with 100 μ L/well of TMB development solution, and incubated for five minutes at room temperature. Color development was stopped with the addition of 100 μ L/well of STOP solution and plates were read at 450nm, once again within 15 minutes of the STOP.

The proteins, IL17Cm and the control IL27m, were biotinylated by adding a 0.5 mg/mL biotin in water stock to a tube with the specific protein at a 6:1 biotin-to-protein ratio. Upon biotin addition, tube was vortexed, spun, covered with foil (to shield from light), and incubated for 45 minutes at room temperature on micromix machine. The biotinylation was stopped with 50 μ L of a 2M glycine stock.

Hybridoma Fusion

The preliminary screening was used to pick the mice showing significant antibody titer for use in the hybridoma fusion. The animals chosen were 18547-18550, the mice immunized with IL17Cm conjugated with BSA and immunomodulators GM-CSF and CPG. These animals were sacrificed and their spleen cells and lymph nodes were harvested for the hybridoma fusion and placed in a 50mL tube with about 15mL of sterile

lymphocyte preparation medium (LPM). The lymphocyte preparation medium is simply a solution consisting of Iscove's Modified Dulbecco's Medium (IMDM) with a 1X concentration of penicillin G sodium: streptomycin sulfate.

In preparation for the fusion, the spleen and lymph nodes were resuspended in 5 mL of the LPM and poured into a 35mm Petri dish in order to prepare a single cell suspension. To do this, the spleens and lymph nodes were cut several times and their lymphocytes were liberated until only colorless stromal tissue remained. The cell suspensions were then pooled and filtered through a 40 μ cell strainer into a 50mL tube; residual cell clumps were pressed through strainer, which was continually being rinsed with fresh LPM. The filtered cell suspension, consisting of the cells taken from all four animals used, was then divided up between Johanna Harshman, Ursula Garrigues, and myself (Drew Calhoun), for individual workup. The cell suspension aliquot I received was then centrifuged for 10 minutes at 1100rpm, the supernatant aspirated, and the pellet resuspended in 25mL of LPM. A 400 μ L 1:10 dilution of this suspension in LPM was then made, followed by a 1:2 dilution of this mix in 2% acetic acid. This 80 μ L sample was used to count the lymphocytes available using a hemocytometer on an inverted microscope.

Also in preparation for the fusion, mouse myeloma cells (Ag8s) were grown in complete IMDM, which consists of IMDM with 10% fetal clone I serum, 1X L-glutamine, and 1X penicillin-streptomycin, as was used before. The cells were constantly maintained at a stage of log-phase growth, at a density of 2-4 x 10⁵ cells/mL, ideal for fusion. When the spleen cells were counted and ready for fusion, these cells were checked for purity and viability, and counted, too.

The fusion protocol required a 2:1 ratio of spleen cells to myeloma cells to mix together, so the spleen cells were counted and the total number of spleen cells available for fusion was determined to be 6.9×10^7 cells. The myeloma count showed a density of 3.9×10^5 cells/mL and because half of the total available spleen cells, 3.45×10^7 cells, were necessary, 88.5mL of the myeloma suspension was used. The 88.5mL of Ag8 cells were centrifuged at 1100rpm for 5 minutes, supernatants were removed, and pellets were resuspended and pooled in a total volume of 25mL of LPM, which was then added to the tube containing the spleen cell suspension.

The new cell suspension containing spleen/lymph node cells and myelomas was then centrifuged at 1100rpm for 10 minutes at room temperature and the supernatant media was completely removed. The pellet was broken and the cells resuspended by continual hard tapping for about 2 minutes until a fine suspension was acquired. The tube was then placed in a 37 C° water bath and 1mL of pre-heated 37 C° 50% PEG (polyethylene glycol) solution was added dropwise over 1 minute while stirring. Another minute of stirring alone ensued, and this was followed by the logarithmically increasing addition of 25mL of warmed, complete IMDM over the course of the next 5 minutes, all while stirring and on the 37 C° water bath. The contents of the tube were mixed well by gentle inversions of the tube and then incubated an additional 10 minutes in the 37 C° water bath. After this incubation, the cells were centrifuged at 800rpm for 5 minutes, the media aspirated, and the cells were resuspended in a total of 138mL of fusion media to achieve a 7.5×10^5 cells/mL density. This density was used so that the cells could be plated at a density of 1.5×10^5 cells/well with 200 μ L being used per well; this plating density had been previously optimized by ZymoGenetics researchers to promote viable

hybridoma growth. The fusion media consisted of complete IMDM with the addition of 10% hybridoma cloning actor and 1X HAT (hypoxanthine-aminopterin-thymidine) solution. My individual workup yielded a total of seven 96-well plates with fusion samples, which were added to those plated by Johanna Harshman and Ursula Garrigues, for a total of 26 fusion plates; the fusion was numbered “346.” The hybridoma fusion #346 cells were then maintained and fed with fresh fusion media for the following week before screening for antibody-producing hybridomas.

Screening for Hybridomas

The supernatants of the 26 plates obtained from the fusion were initially screened for antibody production using identical Direct and Capture ELISA protocols as were described before, however, the IL17Cm antibodies in this case came from the supernatants of the 96-well plates. A heart bleed from the sacrificed mouse #18547 was used as a positive control, while normal mouse serum (NMS) served as a negative control. The Capture ELISA was a 26-plate assay performed by robotics and due to extremely high background (in this case and several subsequent ones) the results were compromised and no longer applicable. The direct ELISA was performed in two sessions, initially on plates 9-11 and 20-26, then on plates 1-8 and 12-19. The direct ELISA was followed by a visual screen for growing hybridomas in the potential positive wells. The primary screen yielded 42 positive wells with viable hybridomas and these individual hybridomas were transferred to 24-well plates and grown in 2mL of similar culture medium, but with HT (hypoxanthine-thymidine) selection. After about a week’s worth of growth and feeding,

the supernatants of these samples were removed for screening and the cells were resuspended in 1mL and frozen for potential later use.

The 42 hybridoma samples were then screened again for viable, growing hybridomas with significant anti-IL17Cm monoclonal antibody production. This screening was also carried out by an identical direct ELISA on the supernatants obtained from the 24-well plates. This confirmation screen showed that 12 of the 42 samples remained as growing, antibody-producing hybridomas.

Final Screening for Neutralization and Non-Blocking Ability

The supernatants of the 42 fusion samples from the 24-well plates, especially the 12 identified with remaining monoclonal antibody production, were further screened for their ability to neutralize the interaction between IL17Cm and IL17RE. The samples were also tested for the ability of the antibodies to bind to the protein without blocking its interaction with IL17RE. Such antibodies are known as non-blocking antibodies.

EC50 Determination

First, the concentration of protein (IL17Cm) needed to bind the soluble receptor (IL17RE) at least 50% of the time, also known as the EC50, was found. This was done using a capture ELISA in which the coating antibody, a goat anti-human Fc-specific IgG, was diluted to 0.5µg/mL in ELISA A buffer and coated 100µL/well. The plates were then sealed and incubated at 4 C° overnight. The plates were then washed two times with 250µL/well of ELISA C, flicked to empty, and blocked with 200µL/well of 1% BSA/Tween-20. After an hour of incubation at room temperature, the plates were flicked to empty and the soluble form of the IL17RE receptor was plated at 0.5µg/mL using

100 μ L/well. After another hour of incubation at room temperature, the plates were washed two times with 250 μ L/well of ELISA C and biotinylated IL17Cm solutions were then plated. The IL17Cm was biotinylated in the same way as described previously and was then diluted to 1 μ g/mL in ELISA B buffer. Four separate wells in the top row were plated with 100 μ L/well of this concentration and a ten-fold serial dilution was performed on subsequent rows, so that biotinylated-IL17Cm concentrations ranged from 1 μ g/mL to 1×10^{-7} μ g/mL in the bottom row. After the plating of the biotinylated-IL17Cm, plates were incubated for 1 hour at room temperature in the dark, washed two times with 250 μ L/well of ELISA C, plated with 100 μ L/well of SA-HRP diluted in ELISA B (1:2000), and incubated for another hour in the dark. The plates were then washed five times with 250 μ L/well of ELISA C, plated 100 μ L/well with TMB development solution, incubated for 5 minutes, and then “stopped” with 100 μ L/well of Stop Solution. As before, plates were read at 450nm within 15 minutes of the stopping of color development.

Neutralization Screening

After the EC50 for the IL17Cm binding to the IL17RE was determined, the neutralization assays were performed. First, heart bleeds from the immune mice, 18547, 18549, and 18550 were assayed for neutralizing ability. Then, the 42 fusion samples (previously determined) were assayed for their neutralizing ability. To do this, another capture ELISA was performed, and it was identical to the EC50 Capture ELISA protocol up until (and including) the plating of the soluble IL17RE.

In the assay of the immune mouse sera, 120 μ L of a 1:10 dilution of the heart bleeds of the three mice was plated, and a ten-fold serial dilution was performed. The positive control, IL17RE at an initial concentration of 10 μ g/mL, and the negative control,

normal mouse serum diluted 1:10, were also serially diluted as described. This dilution plate map is shown below.

Table 1.2: Sera Dilution Plate Map

	18547	18549	18550	nms	IL17RE
A	1:10	1:10	1:10	1:10	10 ug/mL
B	1:100	1:100	1:100	1:100	1
C	1:1000	1:1000	1:1000	1:1000	1:10
D	1:10000	1:10000	1:10000	1:10000	1:100
E	1:100000	1:100000	1:100000	1:100000	1:1000
F	1:1000000	1:1000000	1:1000000	1:1000000	1:10000
G	1:10000000	1:10000000	1:10000000	1:10000000	1:100000
H	1:100000000	1:100000000	1:100000000	1:100000000	1:1000000

Note: Each column represents two lanes of wells on the 96-well plate.

At this point, 60 μ L of these dilutions was mixed with 60 μ L of 0.01 μ g/mL bio-IL17Cm, resulting in a final concentration of 0.005 μ g/mL for IL17Cm, which was the determined EC50. The mix was then incubated for 45 minutes at 4 C° to allow time for it to complex. The complex was then moved onto the assay plate after the soluble receptor had been plated as the previous capture ELISA describes. In the last two lanes of the assay plate, only bio-IL17Cm at .005 μ g/mL was plated. The plate was then incubated for 1 hour at room temperature, washed two times with 250 μ L/well of ELISA C, and SA-HRP (diluted 1:2000 in ELISA B) was plated at 100 μ L/well. Plates were then incubated another hour at room temperature and washed five times with 250 μ L/well of ELISA. Finally, the TMB color development, stopping, and reading at 450nm was carried out exactly as before.

In the assay of supernatants of Fusion 346, the capture ELISA protocol was carried exactly the same, but the complexes added were simply changed. The 60 μ L of bio-IL17Cm was complexed with 60 μ L of the 42 samples from fusion 346, fusion 336.8, and dilutions of mouse 18549's heart bleed, normal mouse serum, and the soluble receptor according to the plate map shown below. BSA served as a negative control to establish the background level for this assay.

Table 1.3: Fusion 346 Supernatant Screening Plate Map

1	2	3	4	5	6	7	8	9	10	11	12
13	14	15	16	17	18	19	20	21	22	23	24
25	26	26	27	28	29	30	31	32	34	35	36
37	38	39	40	41	42	336.8	BSA	BSA	BSA	BSA	BSA
BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
1:2E3	1:2E3	1:2E3	1:2E3	0.5	0.5	BSA	BSA	BSA	BSA	BSA	BSA
1:2E4	1:2E4	1:2E4	1:2E4	0.05	0.05	BSA	BSA	BSA	BSA	BSA	BSA
1:2E5	1:2E5	1:2E5	1:2E5	0.005	0.005	BSA	BSA	BSA	BSA	BSA	BSA
18549		NMS			IL17RE		Background				

After these samples were complexed with bio-IL17Cm, they were plated, developed, and read at 450nm, as described in the previous assay.

Non-blocking Antibodies

The screening for non-blocking antibodies was carried out using a slightly modified capture ELISA. First, the goat anti-human Fc-specific IgG was coated at 0.5 μ g/mL with 100 μ L/well, as before. Plates were then sealed, incubated 1 hour at 37 C°, washed two times with ELISA C, blocked with ELISA B, incubated for another hour at room temperature, and then flicked to empty. At this point, the soluble form of IL17RE was

plated 100 μ L/well at a 0.5 μ g/mL concentration and the plate was again incubated at room temperature for 1 hour. This was followed by the plating of 100 μ L/well of the ligand, IL17Cm, at 0.5 μ g/mL, another hour of room temperature incubation, and two ELISA C washes. The antibody samples from Fusion 346 and the controls were then plated according to the same plate map shown in **Table 3**. Plates were then incubated for 1 hour at room temperature before another two washes with ELISA C. At this point, the detection antibody, a goat anti-mouse Fc-specific IgG linked to HRP, was diluted 1:5000 in ELISA B and plated at 100 μ L/well. The plate was then incubated for 1 hour at room temperature, washed five times with ELISA C, and color development with TMB and stop solution was carried out, as before. Plates were again read at 450nm within 15 minutes of the stopping of color development.

Results

Preliminary Screening of Immune Mouse Sera

Direct ELISA results

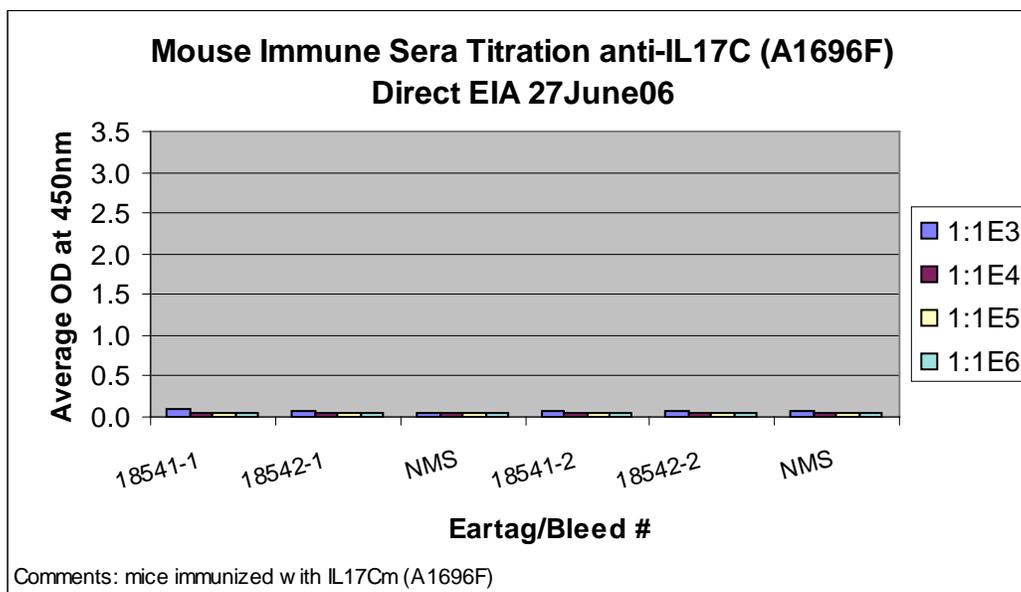
The absorbance results of the preliminary screening of the mice using the direct ELISA method can be seen in the sample table below of the 1st plate of the IL17Cm experimental setup. The other plate of the experimental setup, associated with mice 18546-18550 showed increased absorbance readings across the spread of dilutions, a result which will be shown by graph shortly. Those plates coated with BSA and IL27 showed similar absorbances, both equal to that expected for a normal background level.

Table 2.1: Plate 1 of IL17Cm-coated Direct ELISA (preliminary screen)

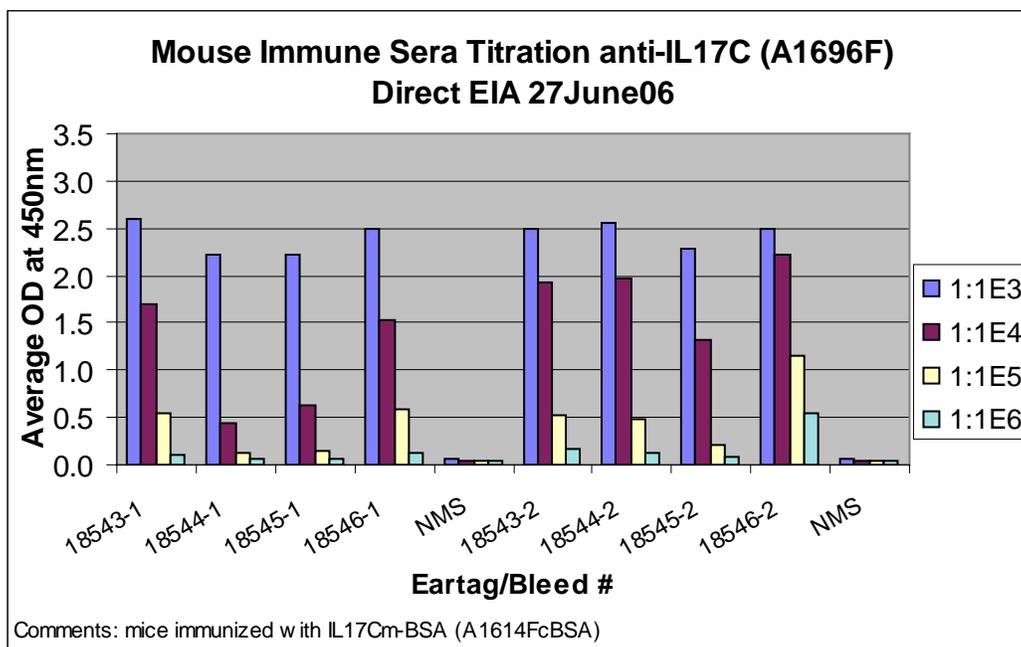
	18541-1	18541-1	18542-1	18542-1	18543-1	18543-1	18544-1	18544-1	18545-1	18545-1	NMS	NMS
1:1E3	0.085	0.117	0.078	0.074	2.614	2.588	2.204	2.256	2.205	2.245	0.059	0.049
1:1E4	0.05	0.047	0.047	0.044	1.834	1.55	0.354	0.545	0.6	0.647	0.045	0.044
1:1E5	0.047	0.042	0.043	0.042	0.562	0.52	0.112	0.13	0.135	0.153	0.047	0.045
1:1E6	0.042	0.041	0.041	0.041	0.109	0.087	0.053	0.053	0.057	0.053	0.042	0.043
1:1E3	0.058	0.065	0.073	0.069	2.471	2.527	2.567	2.535	2.273	2.281	0.051	0.073
1:1E4	0.047	0.048	0.05	0.047	1.913	1.939	1.958	1.977	1.428	1.215	0.046	0.045
1:1E5	0.047	0.045	0.044	0.048	0.538	0.503	0.531	0.431	0.225	0.212	0.045	0.049
1:1E6	0.049	0.049	0.045	0.052	0.148	0.18	0.13	0.126	0.093	0.069	0.045	0.048
	18541-2	18541-2	18542-2	18542-2	18543-2	18543-2	18544-2	18544-2	18545-2	18545-2	NMS	NMS

The raw data from the experimental setup of the Direct ELISA screening of the mice was reformatted into a more effective representation and these graphs are shown below.

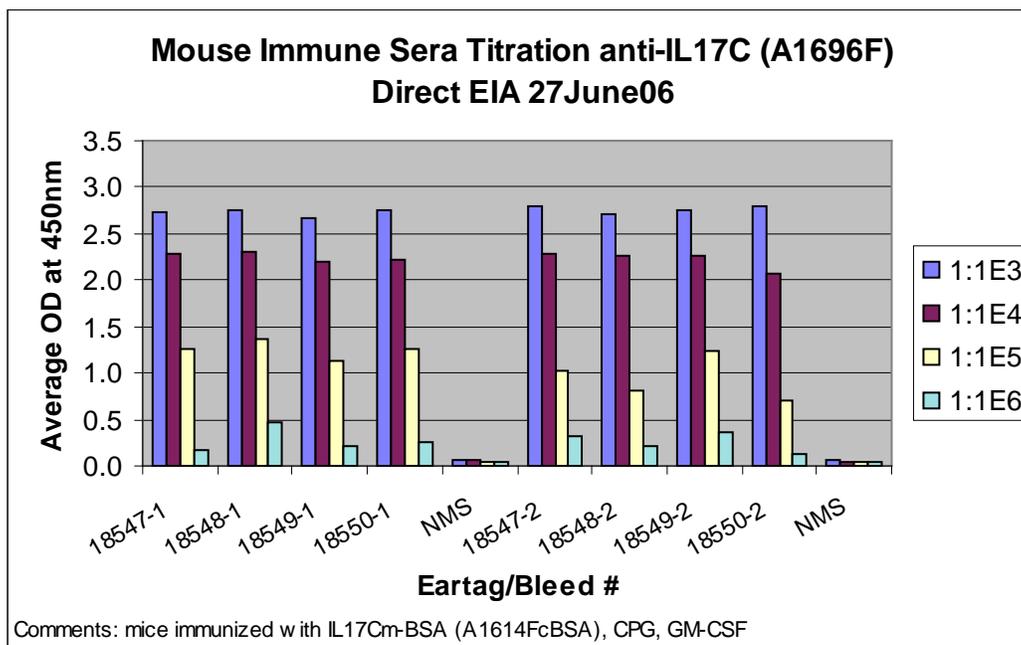
Graph 1: Mouse numbers 18541-18542 ~ Direct ELISA



Graph 2: Mouse Numbers 18543-18546 ~ Direct ELISA



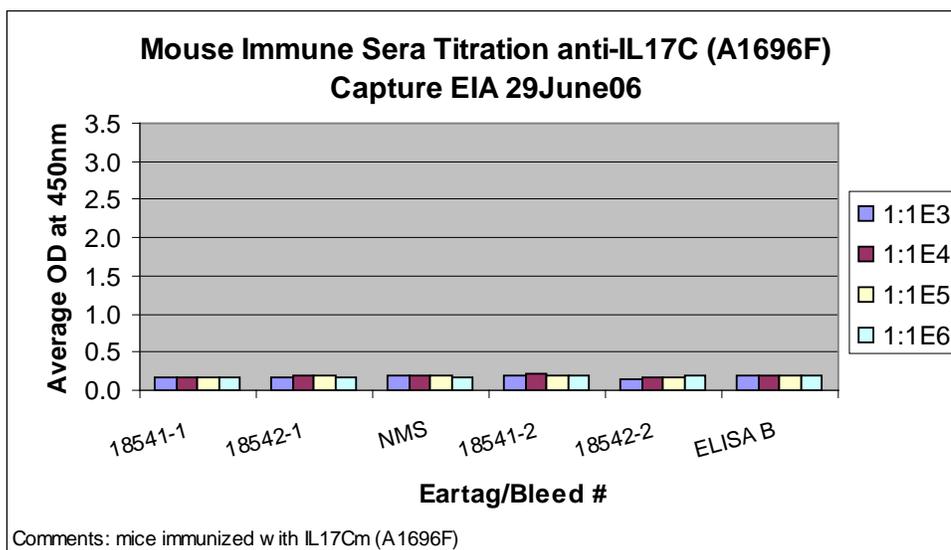
Graph 3: Mouse Numbers 18547-18550 ~ Direct ELISA



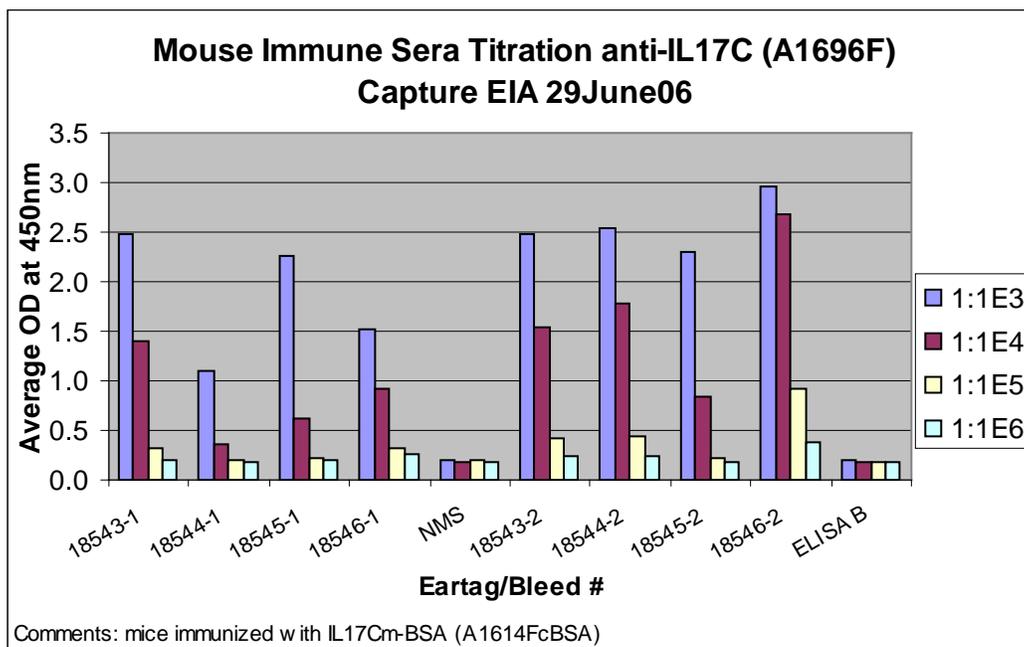
Capture ELISA results

The results from the preliminary screening using the Capture ELISA method showed a similar distribution of antibody titers. Notably, the background absorbance was higher in this method, but not to a point of any consequence. The graphs are shown here, as before.

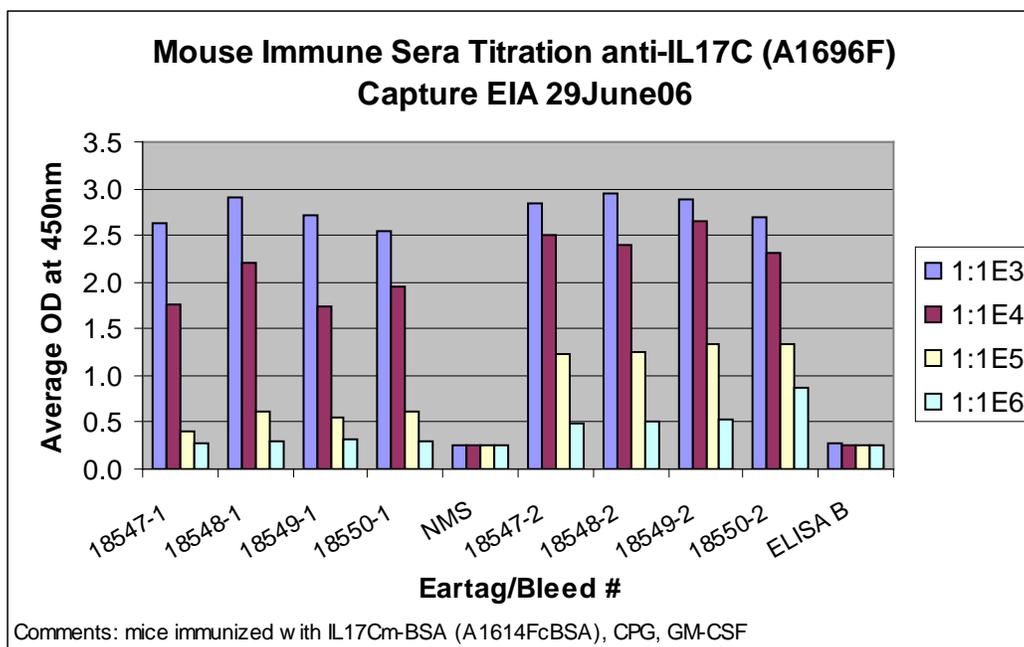
Graph 4: Mouse Numbers 18541-18542 ~ Capture ELISA



Graph 5: Mouse Numbers 18543-18546 ~ Capture ELISA



Graph 6: Mouse Numbers 18547-18550 ~ Capture ELISA



Based on the results obtained from these ELISA methods, the mice numbered 18547-18550 were sacrificed for the hybridoma fusion.

Screening for IL17Cm Antibody-Producing Hybridomas

Primary Screening

The 26 plates obtained from the fusion process were screened for antibodies using the same Direct and Capture ELISA methods as before. The results for the Capture ELISA, however, showed an extremely high background level to the point where almost no positive wells were discernible. In fact, only fusion sample 346.1 was determined to be positive at an absorbance of 1.077 at 450nm. The Direct ELISA screening of the 26 plates proved to be much more useful and 50 potential positive wells were determined. Plate #25 from the assay is shown below as a sample. The 50 possible positive wells are highlighted in orange on the table and these wells were screened visually under the microscope for actual viable and growing hybridomas. Growing hybridoma colonies were visible in 42 out of these 50 wells. The striked-through highlighted boxes within the sample plate shown below represent the potential positives in which no growing hybridomas were observed.

Table 2.2: Plate 25 of Direct ELISA (primary screening of 96-well fusion samples)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.136	0.107	0.161	0.087	0.175	0.405	0.451	0.166	0.194	0.093	0.123	0.173
B	0.077	0.292	0.093	0.110	0.228	0.088	0.088	0.219	0.158	0.279	0.088	0.102
C	0.115	0.130	0.102	0.079	0.090	0.087	0.088	0.079	0.094	0.104	0.097	0.099
D	0.118	0.269	0.183	0.279	0.069	0.086	0.188	0.078	0.097	0.325	0.122	0.100
E	0.142	0.074	0.093	0.090	0.073	0.123	0.143	0.070	0.328	0.102	0.095	0.135
F	0.094	0.092	0.085	0.106	0.068	0.349	0.069	0.086	0.113	0.155	0.094	0.161
G	0.176	0.097	0.099	0.103	0.106	0.251	0.123	0.119	0.107	0.088	0.185	0.114
H	0.349	0.120	0.135	0.233	0.180	0.100	0.091	0.191	0.149	0.100	0.097	3.894

The 42 wells showing viable, growing, and antibody-producing hybridomas were transferred to 24-well plates and the supernatants of these were analyzed in a similar confirmation screen using the direct ELISA method. Samples from fusion 335 were also included as negative controls and are highlighted in pink. Cells highlighted in blue are also controls consisting of only BSA in place of the antibody samples; these values represent the background level of the assay. Samples highlighted in orange represent the 12 positive wells that were observed, as is indicated by their increased absorbance value, while the cells in yellow represent the remainder of the 42 samples of Fusion 346, which showed no anti-IL17Cm antibodies upon this confirmation screen of the supernatants taken from the 24-well plates.

Table 2.3: Confirmation Screen (Direct ELISA of 24-well plate supernatants)

Fusion 346	1	2	3	4	5	6	7	8	9	10	11	12
1-12	2.988	0.603	0.050	0.062	0.071	0.083	0.091	2.601	2.849	1.209	0.411	0.093
13-24	0.083	0.159	0.094	0.050	0.124	0.052	0.053	0.136	0.762	0.622	2.410	0.520
25-36	0.106	0.071	0.066	2.572	0.050	0.098	0.065	0.054	0.051	0.179	0.066	0.251
37-42	0.119	0.157	0.118	0.050	0.113	0.099	0.049	0.047	0.053	0.052	0.050	0.050
	0.058	0.055	0.055	0.054	0.052	0.053	0.054	0.055	0.054	0.053	0.058	0.054
	0.052	0.049	0.051	0.050	0.049	0.049	0.049	0.047	0.050	0.051	0.051	0.049
	0.053	0.052	0.051	0.051	0.053	0.052	0.051	0.053	0.052	0.051	0.052	0.054
	0.052	0.057	0.054	0.055	0.050	0.051	0.056	0.058	0.055	0.051	0.051	0.050

These results showed that 12 of the Fusion 346 samples still were viable, growing, and producing monoclonal antibodies against IL17Cm. These were the samples numbered 1, 2, 8, 9, 10, 11, 21, 22, 23, 24, 28, and 36 of the hybridoma fusion line #346. All 42 samples were still analyzed for their neutralization and non-blocking abilities.

Final Screening for Neutralization and Non-Blocking Ability

EC50 Determination

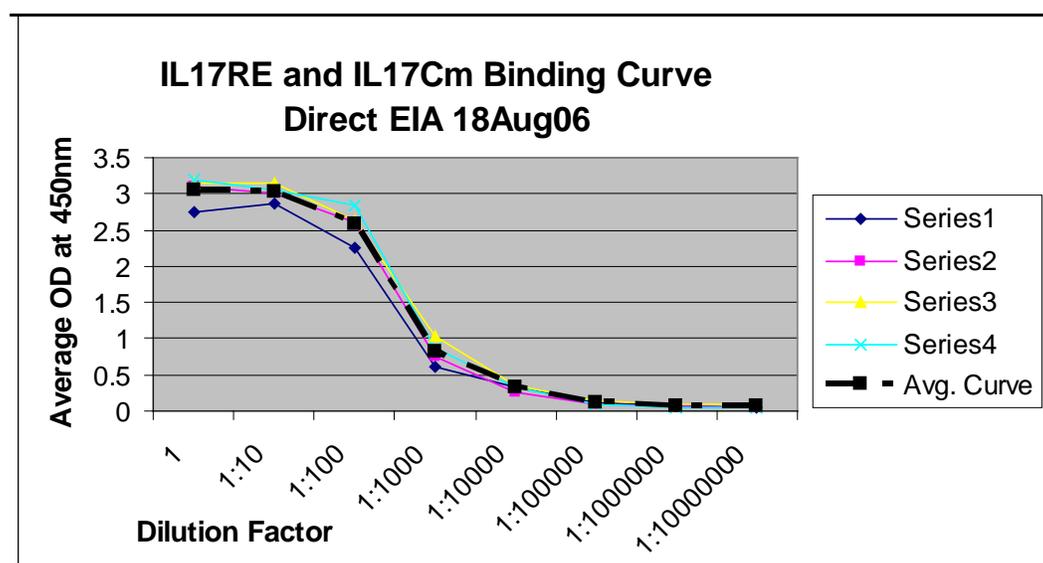
The EC50 was determined to be 0.005 μ g/mL after the values from the serial dilution of the 4 separate lanes of wells were averaged and the midpoint of the binding curve was determined. The results from the absorbance readings at 450nm are shown in **Table 4** below. The background level for this Capture ELISA was about 0.05.

Table 2.4: EC50 Determination Results (OD at 450nm)

IL17Cm Conc.	1	2	3	4
1	2.7427	3.0919	3.1379	3.1967
0.1	2.876	3.0144	3.1473	3.0426
0.01	2.2508	2.613	2.6401	2.8448
0.001	0.6011	0.7517	1.0396	0.8678
0.0001	0.3196	0.2665	0.3622	0.3234
0.00001	0.1283	0.0964	0.1421	0.1023
0.000001	0.0802	0.0643	0.0824	0.0585
0.0000001	0.0555	0.0548	0.0843	0.052

The binding curve established from these results is shown in **Graph 7**. The thick, black curve is the average and its midpoint was used to estimate the EC50 of 0.005 μ g/mL.

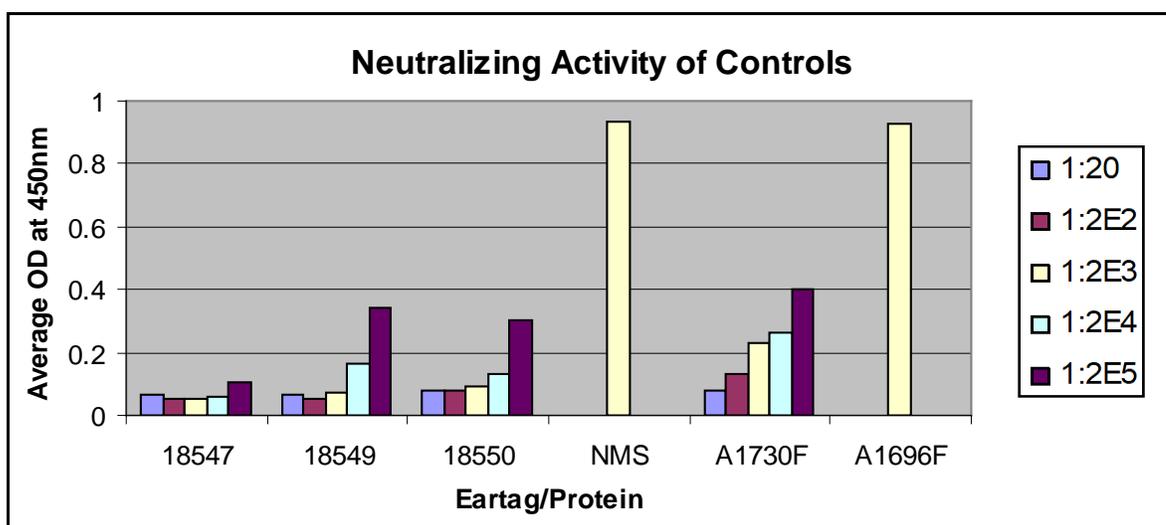
Graph 7: EC50 Binding Curve



Neutralization Screening

The neutralization screening of the immune mouse serum showed significant neutralization at high concentrations and decreased with the 10-fold dilutions. The soluble IL17RE, also known as A1730F, showed similar neutralization characteristics as its concentration decreased with the 10-fold dilutions. The normal mouse serum (NMS) and IL17Cm-only (A1696F) wells were averaged into one value on the graph shown below because they were plated at equal concentrations and showed similar OD readings in the assay.

Graph 8: Immune Mouse Serum Neutralization Screening



The neutralization screening of the supernatants of the 42 samples from Fusion 346, according to the plate map described in the “Materials & Methods,” using SA-HRP as the detection agent showed that 346.1, 346.9, and 336.8 are potential candidates with neutralizing ability. The exact readings of this plate are shown in the table on the next page. The different colors correspond to samples from fusion 346, fusion 336, mouse 18549’s heart bleed, normal mouse serum, the soluble receptor, and BSA-only wells,

which are, once again, in accordance with the previously displayed plate map. White cells represent the fusion samples showing possible neutralization.

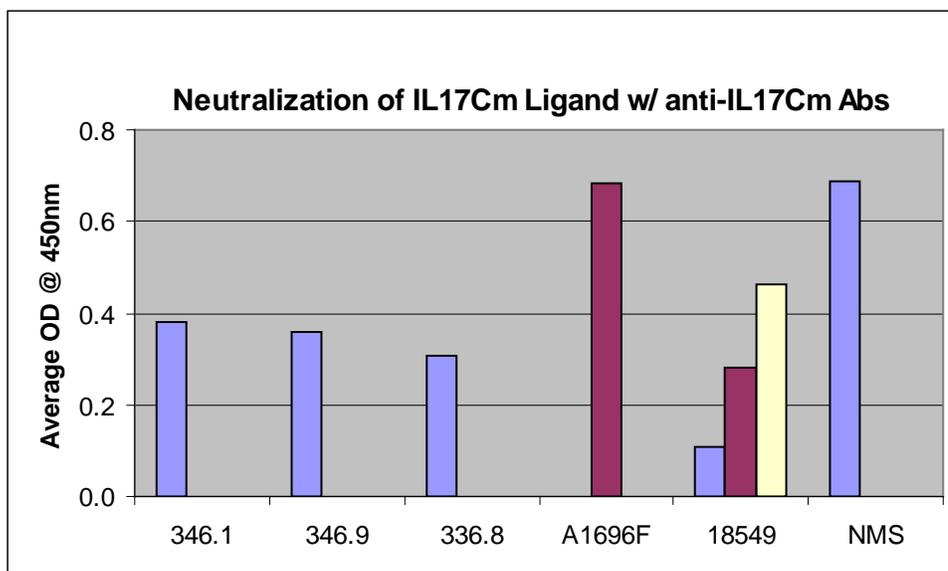
Table 2.5: Neutralization Screening of Supernatants from Hybridoma Fusion 346

1	2	3	4	5	6	7	8	9	10	11	12
0.380	0.882	0.888	0.921	1.056	0.963	0.954	1.032	0.358	0.958	1.073	1.106
0.776	0.910	0.944	0.706	0.933	1.044	0.951	0.998	0.965	0.903	1.036	0.982
0.841	0.964	0.906	0.918	1.047	0.895	0.762	0.955	0.730	0.827	0.976	0.982
0.815	0.910	0.929	0.714	0.892	0.958	0.582	0.738	0.633	0.676	0.683	0.780
0.306	0.063	0.071	0.104	0.080	0.069	0.074	0.098	0.083	0.084	0.104	0.161
0.102	0.119	0.708	0.663	0.155	0.179	0.071	0.093	0.081	0.075	0.102	0.132
0.252	0.314	0.673	0.684	0.293	0.285	0.129	0.146	0.104	0.099	0.150	0.114
0.405	0.517	0.704	0.731	0.440	0.469	0.133	0.189	0.129	0.101	0.259	0.106

The potential positive wells, as compared with the controls, are shown in the graph below.

Note: The soluble receptor control (highlighted in blue above) is not shown here.

Graph 9: Neutralization Screening ~ Potential Positives



Non-blocking Antibodies

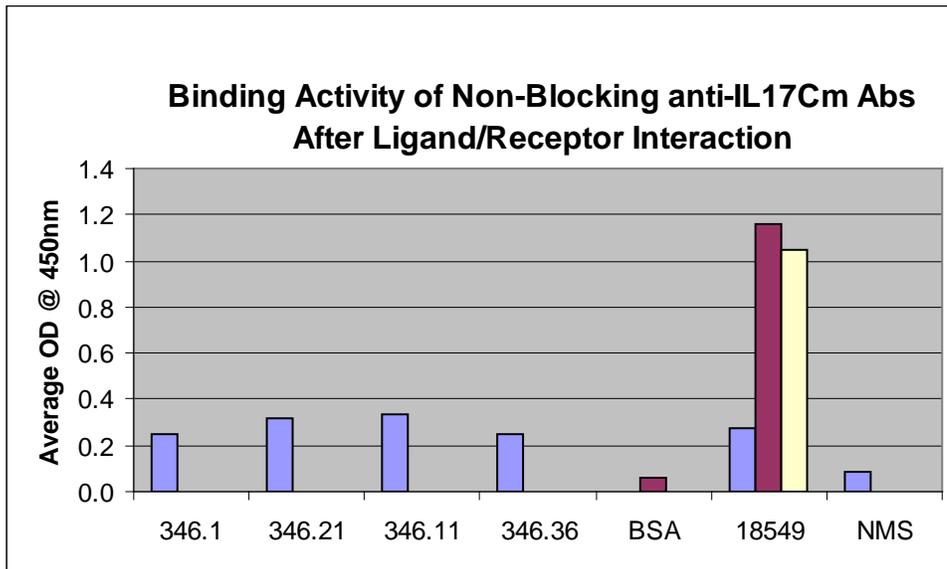
The non-blocking antibodies were detected using a Goat anti-mouse Fc-specific IgG linked to HRP and any heightened absorbance readings would imply that binding of anti-IL17Cm antibodies had bound without interrupting the IL17Cm and IL17RE interaction. The potential non-blockers were determined to be 346.1, 346.11, 346.21, and 346.36 with these results. Even the initial assay data (shown in **Table 6** below) indicated 346.40 as a possible positive, subsequent assays performed by Johanna Harshman showed that this was probably just a false positive and can be disregarded as such. The results from this assay are shown in the table below and, once again, are in accordance with the plate map described earlier in the “Materials & Methods” section.

Table 2.6: Non-blocking Antibodies Screen (*Detection: G α-M IgG Fc-specific-HRP*)

1	2	3	4	5	6	7	8	9	10	11	12
0.246	0.070	0.090	0.073	0.065	0.092	0.086	0.069	0.083	0.074	0.333	0.139
0.054	0.089	0.059	0.053	0.093	0.111	0.074	0.161	0.320	0.098	0.104	0.076
0.066	0.059	0.067	0.102	0.065	0.053	0.058	0.057	0.055	0.061	0.060	0.248
0.066	0.078	0.055	0.313	0.055	0.047	0.054	0.058	0.053	0.056	0.057	0.055
0.092	0.061	0.059	0.057	0.056	0.056	0.056	0.060	0.058	0.061	0.078	0.056
0.257	0.299	0.097	0.067	0.066	0.047	0.050	0.052	0.054	0.050	0.071	0.054
1.103	1.209	0.149	0.060	0.064	0.054	0.059	0.065	0.059	0.147	0.056	0.076
1.022	1.073	0.060	0.060	0.056	0.057	0.366	0.058	0.060	0.396	0.126	0.072

The potential non-blockers, seen in the table above, are also shown in comparison with the controls in the graph provided on the next page.

Graph 10: Non-blocking Antibodies Screening ~ Potential Positives



As can be seen above, samples 346.1, 11, 21, and 36 have significantly elevated values above the background level (BSA) and the negative control of normal mouse serum.

Discussion

Together, the results of the assays imply that, of the Hybridoma Fusion Line 346, 12 samples have potential for anti-IL17Cm monoclonal antibody production; specifically, 346.1, 2, 8, 9, 10, 11, 21, 22, 23, 24, 28, and 36. Of these, 346.1 and 346.9 have potential for neutralizing anti-IL17Cm monoclonal antibodies and 346.1, 11, 21, and 36 have potential for non-blocking anti-IL17Cm monoclonal antibodies. Published results and literature regarding the generation of antibodies against IL17Cm, especially those that neutralize its interaction with IL17RE, are virtually non-existent and while the generation of monoclonal antibodies is by no means a novel idea, those specifically designed against IL17Cm represents an extremely new area of research. Previous fusions, such as Fusion 336, have been carried out by ZymoGenetics to generate such antibodies, but yielded only a limited amount of them.

While the generation of these antibodies looks extremely promising, further tests and research must be done to verify their validity. For example, in the case of 346.1, it showed both neutralizing *and* non-blocking abilities according to the assays, which seems confusing considering the thought that it should be specific for one epitope. This could be for a variety of reasons, not limited to simple experimental error. First, if the antibodies in the supernatant fluid of this sample were not monoclonal, then perhaps we were actually seeing the results of multiple antibodies binding to IL17Cm at different epitopes. Another situation which might produce such a result would be if the antibodies binded on or near the site of IL17Cm biotinylation; such an antibody might disrupt the bond subsequent detection by SA-HRP in the neutralization screen leading to the erroneous conclusion of

its neutralizing ability causing this. Further results, such as initially perceived non-blocking antibodies in 346.40, which contradicted a repeated assay later on, must also be verified through repeated screening to ensure the reliability of the results. Finally, the fact that, of the 12 samples shown to have some sort of anti-IL17Cm antibodies, only 5 were shown to have either non-blocking or neutralizing ability also seems a little bit confusing. However, this could be a likely result if the antibodies from the remaining 7 wells had a relatively low affinity for binding and the stronger affinity for similar epitopes by the newly introduced agents, such as IL17RE, simply displaced them when the complex was plated. Such unexpected results and the potential explanations outlined here, however, can only be verified by further confirmation screening including additional plate-based assays (like those used) and cell-based methods for such screening. These might include transfecting cells with IL17RE and observing the ability of the proposed neutralizing antibodies to block NF- κ B pathway activation, or using a GPI-linked IL17RE receptor to catch the IL17Cm and assay the neutralizing or non-blocking abilities of the antibodies as before. This additional screening, as well as further purification of the supernatants through cloning and subcloning of the promising hybridoma samples, will surely help verify the accuracy of the results gathered and clear up any remaining confusion from unexpected results.

The use of monoclonal antibodies against IL17C as a means of drug therapy remains a possibility. However, such an application will require many more years of research. At this point, though, antibodies developed against IL17Cm, both neutralizing and non-blocking, have the potential to serve as extremely important “proof-of-concept” molecules. The neutralizing antibodies have potential utility as proof of concept

therapeutic molecules and assay reagents, while the non-blocking antibodies can be utilized as reagents in assays such as Sandwich ELISAs. Further research into IL17C, and specifically its interaction IL17RE, especially with the help of anti-IL17C monoclonal antibodies, will be extremely important in understanding its role in the body.

Characterizing this cytokine's functions in the body, especially the extent to which it may carry beneficial roles in the inflammation process, not only has implications for furthering our understanding into the actual mechanisms of the immune system, but also for the possibility of developing therapeutic protein drugs in response to autoimmune inflammatory diseases.

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