Selective Targeting of FIRE/CIRE Dendritic Cell Surface Receptors Using Hybrid Antibodies: A New Methodology to Break Tolerance

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Abstract:

The use of hybrid monoclonal antibodies (mAbs) as a vehicle to deliver a human antigen of interest (hAg) directly to dendritic cells (DCs) is effective at inducing high antibody production. This research was designed to test the efficacy of hybrid mAbs (containing a rat F(ab) portion cloned in frame with a mouse IgG2a Fc portion fused to hAg) to target specifically the FIRE/CIRE C-type lectin endocytic receptors (CLRs) on the surfaces of DCs. In this study, hybrid mAbs were successfully produced, and production was confirmed by Western Blot analysis. The binding specificity of the hybrid mAbs was tested *in vitro* on primary lymphocytes purified from C57BL/6 mice and CHO-K1 cell lines that stably expressed FIRE/CIRE CLRs. Using multicolor flow cytometry, this study found that the hybrid anti-FIRE (6F12) mAb demonstrated specific binding to CHO cell lines; however, the anti-CIRE (5H10) mAbs didn't bind to these cells successfully. The results of the binding affinity test of these mAbs on total lymphocytes were inconclusive due to non-specific binding of the anti-mIgG-FITC secondary antibody, which can be attributed to cross-species reactivity.

The hybrid mAb (anti-FIRE-hAg) generated in this study will be used for *in vivo* studies in the attempt to break tolerance of proteins (hAg) that are highly conserved between mouse and human and are consequently poorly immunogenic. If this antigen delivery mechanism is successful in yielding high antibody titers against hAg, this will hold great promise for application to other vaccine target proteins and Ab drug development in general.

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1. Introduction

1.1. Review of Literature

Dendritic cells (DCs) are considered to be the most potent antigen professional presenting cells able to orchestrate the entire immune response. They have been found to play a pivotal role in the functioning of both innate and adaptive immunity and the body's overall resistance to infection. One of their characteristics is the capacity to stimulate naive lymphocytes and mount specific immune responses (Steinman et al., 2007).

The breadth of DC functions has been attributed to the heterogeneity of the DC cell population. DCs are divided into different subsets based on their localization, surface marker expression, morphology, and functional characteristics (Steinman et al., 2003). The different subsets vary in their ability to process and present antigens (Mellman et al., 2001), which is affected by their maturation state (Figure 1). DC maturation is crucial in regulating tolerance and immunity. Immature DCs are highly efficient in capturing and processing antigens, whereas mature DCs stimulate T cell immunity (Banchereau and Steinman, 1998). Upon contact with a cognate antigen, DCs begin to assume a mature state (Figure 1) that is now ideal for antigen presentation rather than antigen uptake (Caminschi et al., 2009).

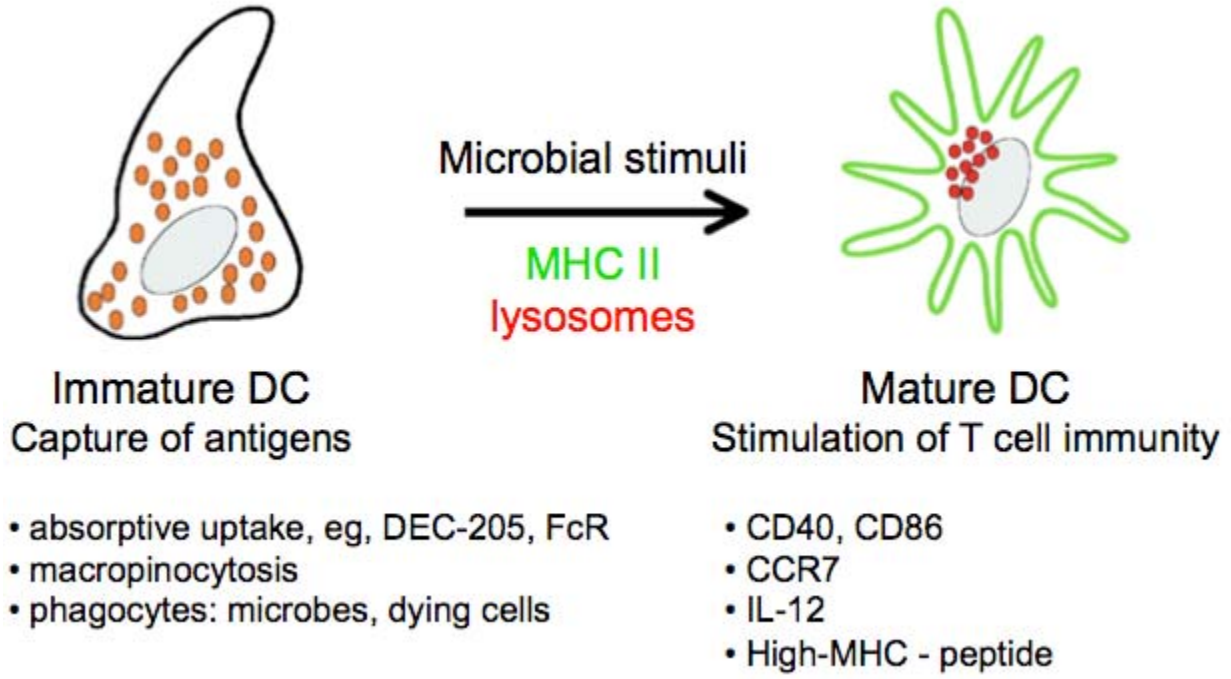

Figure 1: DCs undergo maturation upon contact with and uptake of their cognate antigen through surface receptors, which leads to stimulation of T Cells to mount the immune response (Banchereau and Steinman, Nature 1998, 392:245).

DCs are constantly presenting both foreign and self-antigens to T cells, generating different types of immune responses (Bonifaz et al., 2002). It has been proposed that the ability of DCs to elicit both tolerance and immunity is considered to be essential in the distinction between self and non-self proteins, and may be crucial to the prevention of auto-immunity (Boscardin et al., 2006). To overcome the risk of auto-immunity, it has been suggested that immature DCs induce peripheral tolerance to antigen by the deletion of auto-reactive T cells and/or the induction of regulatory T cells. This occurs in the steady state before DC maturation in the presence of inflammation and infection (Steinman et al., 2003).

Under inflammatory conditions, DCs are prompted to bind to invading pathogens (microbial products) and capture them. Toll-like receptors (TLRs) on the surface of DCs recognize cognate antigens and stimulate the maturation of that cell into a cell with prime antigen-presenting capabilities (Banchereau and Steinman, 1998; Janeway et al., 2002). It has

been recently shown that antigen targeting to C-type-lectin receptors (CLRs), which are specifically expressed by DCs that mediate efficient uptake of proteins or dying cells, can modulate the immune response (Corbett et al., 2005; Hawiger et al., 2001) (Table 1). CLRs are endocytic receptors able to carry antigens into intracellular antigen processing compartments, leading to highly efficient antigen loading to the MHC molecules – a step that is critical for antigen presentation.

CLRs	Cell expression	Ab Isotype	T Cell/MHC	Type of I.R
Dectin-1	$CD8^{-}CD11b^{+}$ & $CD8^{+}$ DCs	IgG2a	High $CD4^{+}$ T Low $CD8^{+}$ T	Th1/Th17
DEC-205	$CD8^{+}$ DC, Lanhgerance DCs, TECs	IgG2a	$CD4^{+}$ & $CD8^{+}$ T cell response (MHCI)	Th1, anti-tumoral
DNGR1/ClecA9	$CD8^{+}$ DCs	IgG2a	CD8+ T & Cross-presentation	Th1, anti-tumoral
DCIRII	$CD8^{-}$ DCs	IgG2b	$CD4^{+}$ T, MHCII	?
FIRE/CIRE	MΦ & $CD8^{-}$ DCs	IgG2a	B cell	Humoral
DC-SIGN (h)	Epith DCs & MoDCs		HIV glycoprot. binding	Th1
ASGRP (h)	Conventional DC	IgG2a	$CD4^{+}$ T	IL-10
Lox1 (h)	Conventional DC	IgG2a	$CD4^{+}$ & $CD8^{+}$ T	Th1
…	…	…	…	…

Table 1: This table denotes the pathways by which antigen uptake by the various C-type lectin receptors (CLRs) on the surfaces of specific DC populations leads to different types of immune responses.

By implementing this antigen targeting method using hybrid antibodies as a vehicle to deliver antigens to DCs *in situ*, there is potentially a way to enhance the humoral immune response and consequently antibody (Ab) production (Corbett et al., 2005). This concept is especially important to consider for vaccination design as it allows scientists to manipulate DCs in order to more effectively manipulate the immune response. Ultimately, the plan is to use this new methodology to develop antibodies against poorly immunogenic proteins that have high homology between mouse and human.

This research is a preliminary step in the attempt to test the efficacy of hybrid monoclonal antibodies (mAbs) generated by "attaching" an antigen of interest "X" to the Fc portion of mAbs and in specifically targeting DCs *in vivo*. The fusion antibodies will specifically target the F4/80-like receptor (FIRE) or the C-type lectin receptor CIRE, which have previously been shown to elicit a high humoral immune response (Corbett et al., 2005). Both CIRE and FIRE are expressed on immature $CD8^-$ mouse DCs. Furthermore, FIRE is expressed moderately on monocytes and macrophages whereas CIRE is expressed on some plasmacytoid DCs in the lymphoid organs (bone marrow, lymph nodes, and spleen) (Corbett et al., 2005) (Table 1).

The objective of this research is to test the binding specificity of anti-FIRE and anti-CIRE mAb (produced by Regeneron Pharmaceuticals [Tarrytown]) to their respective CLRs. However, these hybrid mAbs will eventually be used for *in vivo* CLR targeting in order to generate high Ab titers against a protein of interest.

1.2. Hypothesis

In this study, it is hypothesized that the hybrid mAbs will be successfully produced and bind to CIRE and FIRE CLRs, as visualized by flow cytometry. There are three subcategories of hypotheses that were tested during this experiment. The null hypothesis is also listed.

1. H_1: The hybrid mAbs will be successfully designed and produced.
2. H_2: The hybrid mAbs will specifically bind to the FIRE/CIRE cell surface receptors when incubated *in vitro* with total lymphocytes purified from C57BL/6 mice spleens and lymph nodes.
3. H_3: The hybrid mAbs will demonstrate specific binding to the FIRE/CIRE CLRs that are stably expressed on CHO cell lines.

4. H_0: The hybrid mAbs will not successfully be produced; however, if they are, they will not successfully bind to FIRE/CIRE cell surface receptors.

2. Methods and Materials

2.1. Mice

6-8 week old adult C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and used according to Regeneron Pharmaceuticals institutional guidelines and competition guidelines.

2.2. Generating Anti-FIRE/Anti-CIRE mAb Constructs

The Therapeutic Proteins Department of Regeneron Pharmaceuticals generated the constructs. The protein sequences of the 6F12 (FIRE) and 5H10 (CIRE) variable regions were first submitted for codon optimization [Blue Heron (Bothell, Washington)]. For each variable heavy chain (VH) and variable light chain (VK) sequence, SapI restriction enzyme sites were then placed on either end of the codon optimized sequence, and these genes were synthesized by Blue Heron within their standard pUC vector. Using SapI cloning to avoid the presence of linkers, each VH was cloned into Regeneron's vector onto a mIgG2a constant region, while each variable light chain was cloned onto mouse kappa constant region within a separate vector (Figure 2). Ultimately, sub-cloning the variable regions onto the constant regions resulted in the creation of the constructs encoding for the anti-FIRE and anti-CIRE mAbs, ready for transfection.

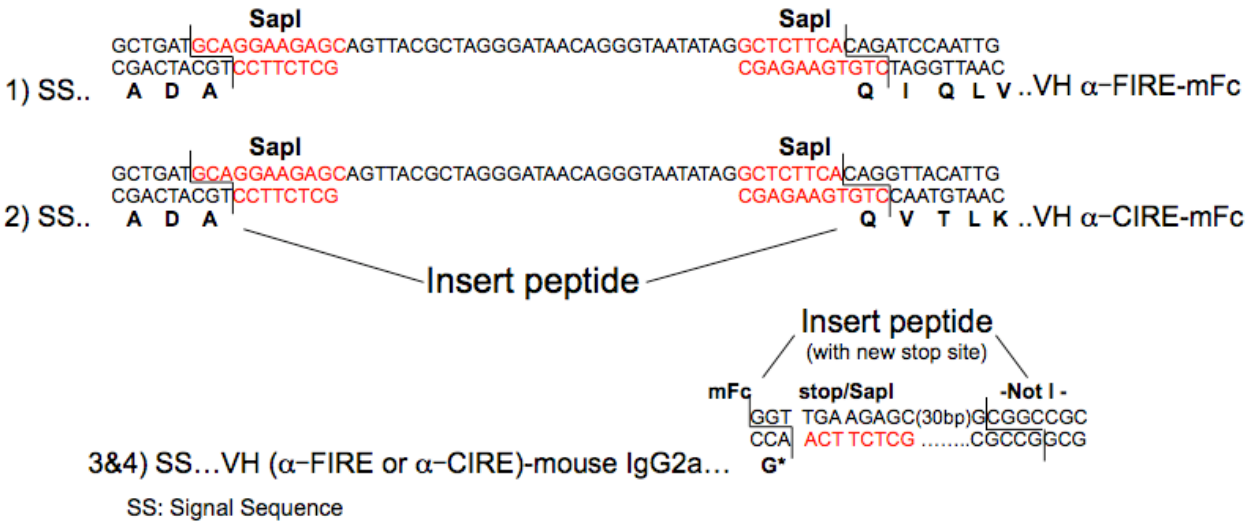

Figure 2: Sap1 sites for peptide insertion are shown in the sequences. Light chain constructs for either anti-FIRE or anti-CIRE can be expressed with N-term or C-term IgG2a fusion vectors for anti-FIRE and anti-CIRE.

2.3. Transfection and Production of Hybrid Antibodies

The fusion mAb constructs were produced by the Therapeutic Proteins department of Regeneron Pharmaceuticals. Hybrid mAbs were generated by transient transfection of Chinese Hamster Ovarian cells (CHO-K1) using Lipofectamine Plus (Invitrogen) protocol. Three separate transfections were done. Sample 1 contained DNA encoding for anti-CIRE (5H10) mIgG2a. Sample 2 contained DNA encoding for anti-FIRE (6F12) mIgG2a. Sample 3 was transfected with 3H7 mock, an empty vector control. Fusion mAbs were harvested from the supernatant 4 days after transfection and purified on 0.22 um Protein G Column filters. The final CHO-K1 supernatant mAb concentrations were estimated to be 0.4 ug/mL for the 6F12 mFc and 1.12 ug/mL for the 5H10 mFc. The concentration of the mock (empty vector) was too low to register. The hybrid mAbs were then submitted to the Protein Development department of Regeneron Pharmaceuticals for Western Blot analysis.

2.4. Western Blot Analysis/SDS-PAGE

Cell lysates from CHO-K1 cells expressing CIRE/FIRE were separated on SDS-PAGE under reducing and non-reducing conditions by the Protein Development Department of Regeneron Pharmaceuticals. The reduced and non-reduced protein samples were prepared as a 1:4 dilution (sample:buffer).The samples were boiled in a heat block at 100°C for 5 minutes and then centrifuged at 13,000 RPM for 1 minute. The gel apparatus (4-20% Tris-Glycine protein gel) was prepared according to standard protocol. After transferring the gel to the gel paper pad, the membrane was taken out of the gel transfer cassette. The membrane was blocked in 10% non-fat milk/TBS-t overnight at 5°C. After blocking, the non-fat milk/TBS-t was washed off with 1X TBS-t. The gel paper pad was then incubated with the primary Ab for 1 hour for probing, washed three times with TBS-t, incubated with the secondary Ab for probing, and washed three times again. The Western Blot film was developed in the Pierce ECL Western Blotting Substrate.

2.5. Binding Affinity

The engineered mAbs were assayed for their binding specificity to FIRE and CIRE CLRs. Spleens and lymph nodes were harvested from the B6 mice. The lymph nodes were crushed while the spleens were chopped with a syringe and digested by incubation with collagenase to liberate DCs from the tissue. Spleens were treated with EDTA to block collagenase action and cells were purified through a strainer. After isolation, cells were incubated with Fc block for 10 minutes at a 1:150 dilution to inhibit non-specific binding to Fc receptors. Purified lymphocytes or CHO cell lines stably expressing FIRE or CIRE (provided by Dr. Ken Shortman of the Walter and Eliza Hall Institute of Medical Research (WEHI), Melbourne, Australia]) were incubated with 6F12 (anti-FIRE), 5H10 (anti-CIRE) or 3H7

(control Ab). DCs were gated based on the expression of mCD11c and MHC class II molecules, using the appropriate staining mAbs. Cells were then washed in 3% FACS buffer. The staining mix (Table 2) containing different combinations of flurochrome-conjugated mAbs were distributed to each well. Cell suspensions were prepared for analysis by flow cytometry. Data were analyzed using FloJo software.

				Ac	finale []°	premix []°	Vol (µL)	Premix (µL)
Premix				+amIgGFitC	1/300	1/300	0.93	vol total
FIRE CIRE	Nb of tubes	7	1					280
			2	Gr1 PE-Cy7	1/300	1/300	0.93	vol mix /tube
			3		0/1	0/1	0.00	40
			4		0/1	0/1	0.00	Buffer vol
			5		0/1	0/1	0.00	
			6	F4/80-PE	1/200	1/200	1.40	267
	X:	1	7		0/1	0/1	0.00	
			8		0/1	0/1	0.00	
			9	CD8 PB	1/100	1/100	2.80	
			10	CD11b A750	1/100	1/100	2.80	
			11	MHCII A700	1/300	1/300	0.93	
			12	CD11c APC	1/200	1/200	1.40	
			13	B220-PerCP	1/200	1/200	1.40	

Table 2: The flurochrome-conjugated staining antibody mix layout used to measure binding affinity of hybrid antibodies to CHO/total lymphocytes as visualized by flow cytometry.

2.6. Flow Cytometry

Flow cytometry is an advanced mechanism used to visualize the binding of Ab to cell surfaces. It implements the principles of light scattering, light excitation, and emission of fluorochromes to yield data that display the binding patterns of variables tested. The LSR II fluorescent activated cell-sorting (FACS) instrument was used to project the binding specificity of the two hybrid mAbs to FIRE/CIRE DC surface receptors respectively by multicolor flow cytometry. These data were projected onto multiple dot-plots/graphs that compared the different fluorochrome-mAb binding and expression patterns to one another. Thus, the evaluation of hybrid Ab cell surface binding to FIRE/CIRE receptors on specific cell populations was performed.

2.7 CHO-K1 Cells from WEHI Melbourne, Australia

CHO-K1 cells used for comparison were provided by Dr. Ken Shortman of WEHI (Melbourne, Australia). (Reference Corbett, et al, 2005 for Ab production protocol).

3. Results

3.1. Generation of Antibody Constructs

To generate hybrid mIgG2a anti-FIRE and anti-CIRE mAbs, cDNA sequences encoding for mouse anti-FIRE, anti-CIRE, or mock were successfully cloned in frame in by means of the Sap 1 restriction enzyme action (Figure 2). The F(ab) regions of the original 5H10 and 6F12 rat Abs were maintained to facilitate proper binding to cognate DC surface receptors; however, the constructs were altered to have the mouse IgG2a Fc portion (Figure 3) in order to avoid production of anti-rat Ab, from cross-species reactivity, upon *in vivo* administration.

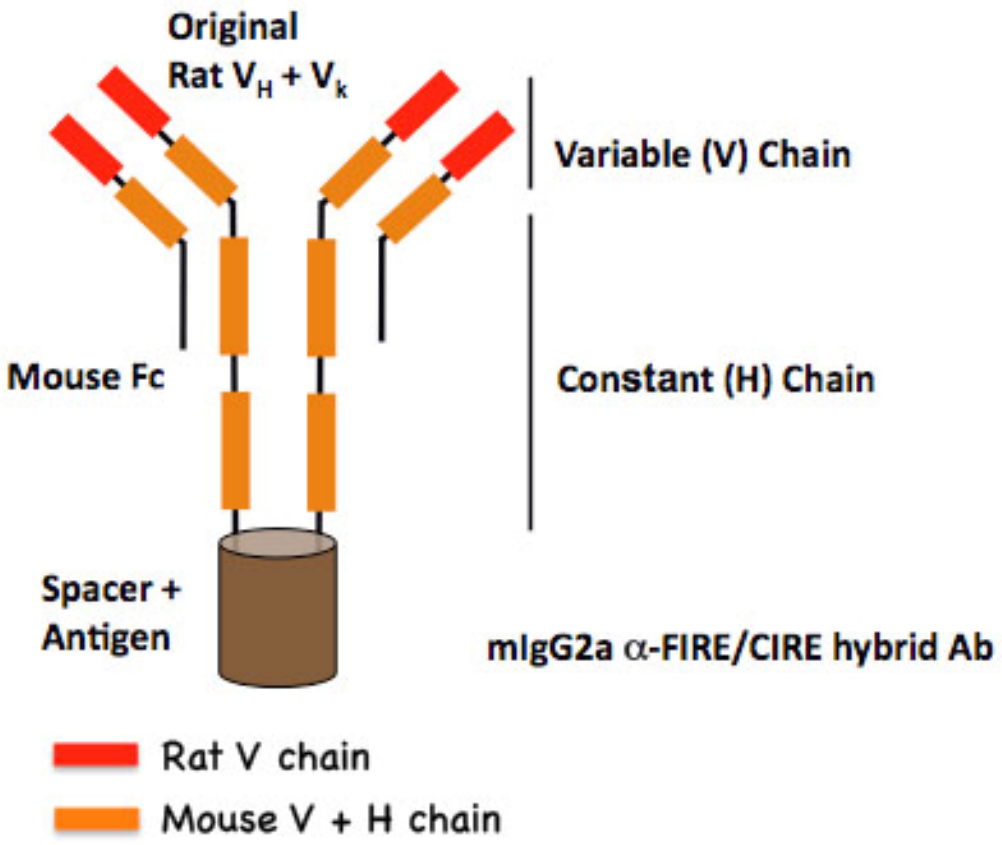

Figure 3: Diagrammatic representation of the hybrid mAbs constructed during this experiment.

3.2. Antibody Production

To produce hybrid mAbs, transient transfection using the CHO-K1 cell line was performed (see methods). Five separate Ab constructs resulted:

1. anti-FIRE
2. anti-FIRE coupled to human antigen of interest (hAg)
3. anti-CIRE
4. anti-CIRE coupled to hAg
5. empty vector (mock)

To determine whether the mFc was successfully cloned in frame to the mF(ab) of the fusion mAb and if the antigen of interest was properly attached to the Fc terminal portion of the

Ab, a Western Blot experiment was designed and conducted (Figure 4). This study found that mAbs coupled to hAg migrated to the expected molecular weight of ~90kD (50kD from the heavy chain and 40kD from the Ag) in reducing conditions and to ~190kD (150kD from the heavy/light chains and 40kD from the Ag) in non-reducing conditions, compared to mAbs lacking the hAg. Therefore, it was concluded that the Regeneron-produced hybrid mAbs were properly generated.

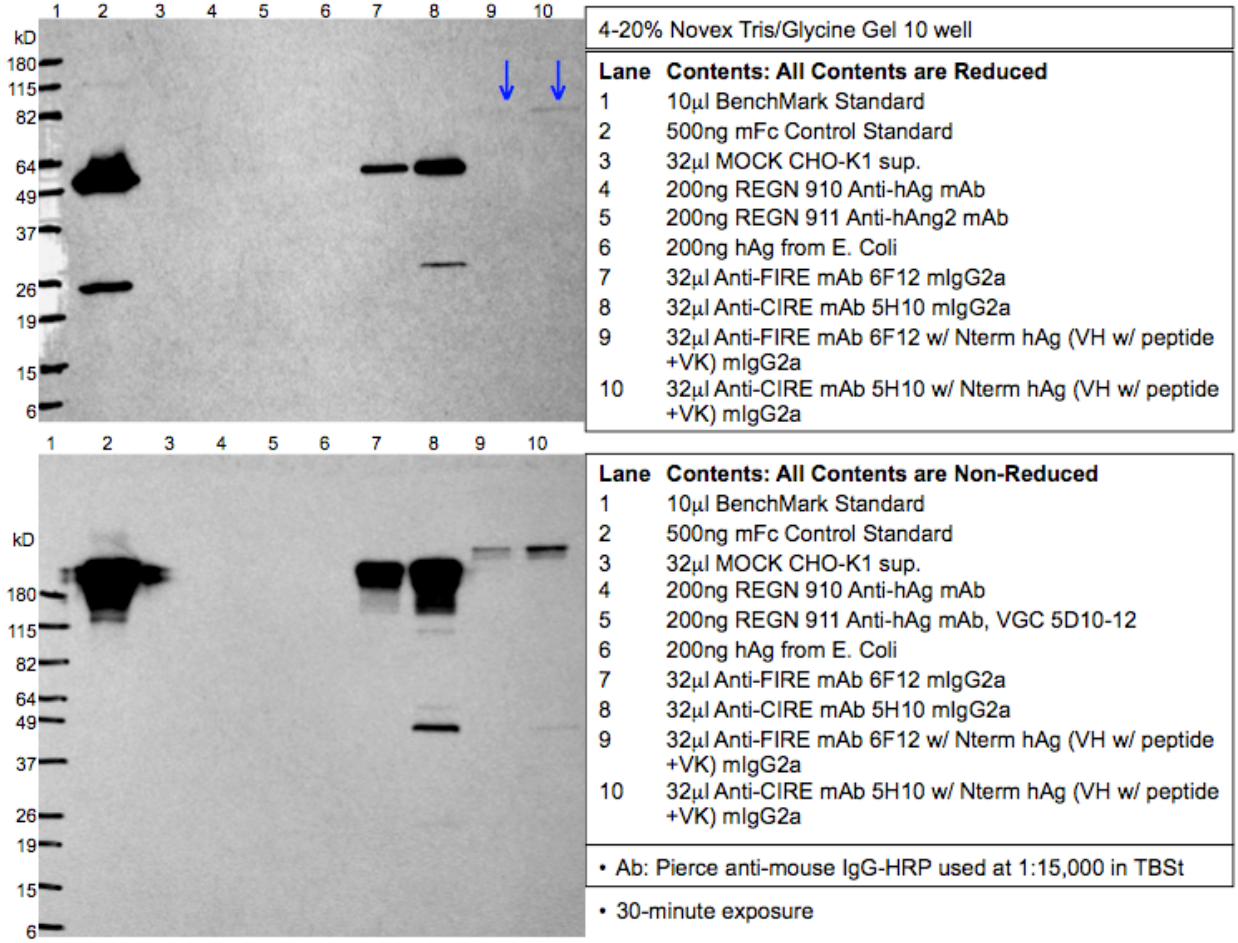

Figure 4: Hybrid mAbs analyzed by Western Blot under reducing and non-reducing conditions. Molecular weights are indicated on the Y-axis.

3.3. Determining the Binding Affinity of mAbs

To evaluate if anti-FIRE/anti-CIRE fusion mAbs were able to bind efficiently to the FIRE and CIRE receptors expressed on the surfaces of primary lymphocytes, flow cytometry

was used. Lymphocyte sub-populations were selectively gated using fluorescent Abs that recognized DCs, macrophages, CD11b cells, granulocytes, or B cells (Figure 5). In this study, it was found that the secondary antibody bound non-specifically to the cell surfaces. Thus, the experiment was inconclusive.

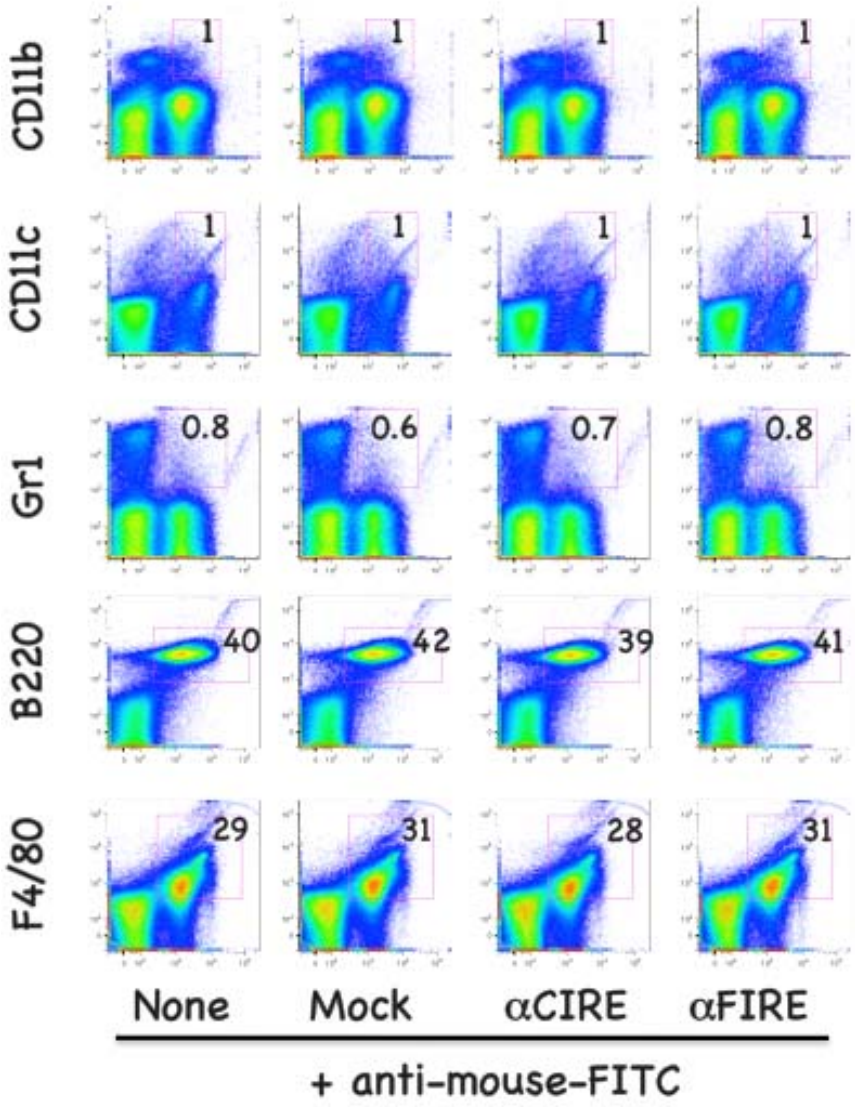

Figure 5: The binding of anti-FIRE and anti-CIRE mAbs to specific lymphocyte sub-populations (B cells, macrophages, granulocytes, and/or dendritic cells) was tested by using fluorochrome-conjugated mAbs specific for certain cell surface markers (B220, F4/80, Gr1, CD11b, and CD11c). Four conditions were tested by using the Regeneron-produced 5H10 (anti-CIRE) mAbs, the 6F12 (anti-FIRE) mAbs, the mock, and a condition with no primary Ab. In all conditions, a secondary Ab (anti-mIgG-FITC) was used to evaluate cell surface binding. An isotype Ab was used as an additional control (data not shown).

3.4. Testing FIRE/CIRE Receptor Binding Affinity Using CHO Cell lines

Previously, in this study, it was found that the anti-mIgG2a-FITC secondary Ab demonstrated non-specific binding to the surfaces of primary mouse splenocytes. Another alternative to test hybrid mAb binding was to use CHO cells expressing CIRE or FIRE CLRs. It was found that the Regeneron-produced anti-FIRE (6F12) mAb was binding to 15% of CHO cells as compared to the original rat-anti-FIRE (WEHI 6F12) construct that showed 30% binding (Figure 6). In contrast, anti-CIRE (5H10) mAbs were unable to efficiently bind to CHO cells, compared to the 5H10 WEHI Abs that showed 27% binding (Figure 6). Therefore, we concluded that it is necessary to re-design and re-produce the anti-CIRE hybrid mAbs in order to yield efficient binding to the CHO cells. In addition, a larger quantity of anti-FIRE-hAg mAb will be produced in order to immunize animals and evaluate if this new methodology will produce high titers of Abs against the hAg of interest.

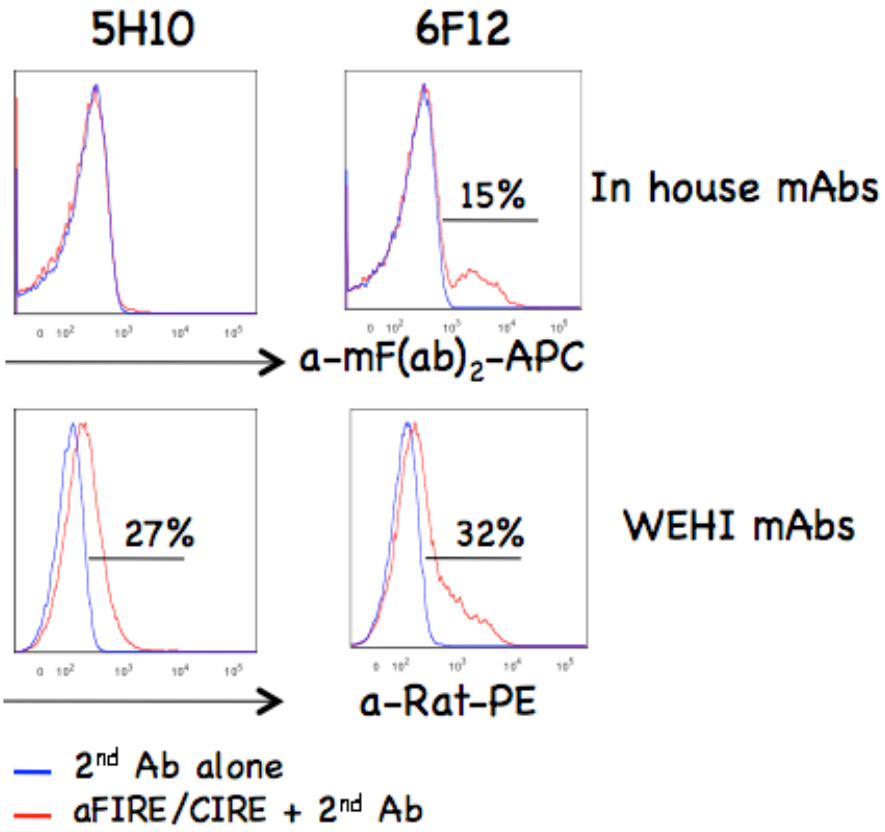

Figure 6: The binding of 5H10/6F12 WEHI rat Abs was compared to the binding of the Regeneron-produced mIgG2a hybrid mAbs to FIRE/CIRE CLRs, which are stably expressed on the surfaces of CHO cell lines (CHO C32 stably express CIRE and CHO F6 stably express FIRE).

4. Conclusions and Discussion

It has been a challenge to generate antibodies against proteins that are highly conserved between species. Therefore, the implementation of a new methodology that uses hybrid mAbs as an antigen delivery vehicle to target dendritic cells *in situ* and enhance the humoral immune response has become a growing area of study (Hawiger et al., 2001; Boscardin et al., 2006; Corbett et al., 2005). This research was a fundamental preliminary step towards the ultimate goal of inducing humoral immunity to antigen "X" by means of immunizing mice with the fusion mAb constructs that were generated during this study. It is expected that this specific targeting mechanism will allow scientists to break tolerance to proteins that are poorly immunogenic (they

don't generally illicit an immune response as they are recognized as self antigen rather than foreign). In this study, the hybrid mAb constructs were designed, produced and tested for binding to FIRE/CIRE CLRs. Anti-FIRE (5H10) mAbs proved to be specific for the CHO-K1-FIRE expressing cells, while anti-CIRE mAbs didn't bind to CHO cells.

There are many factors that could have caused the non-specific binding that occurred upon the incubation of the anti-FIRE or anti-CIRE Abs with the splenocytes. In this study we found that the secondary Ab (rat-anti-mouse IgG2a conjugated to FITC) was binding non-specifically to the cell surfaces of the primary lymphocyte sub-populations. Therefore, this experiment was inconclusive in evaluating the ability of the hybrid Abs to bind to primary lymphocytes. Based on this result, the next step is to use biotinilated hybrid mAbs in order to avoid the non-specific binding from the secondary Ab due to species mismatch.

It has been previously shown that targeting the FIRE/CIRE DC CLRs on the surfaces of $CD8^-$ DCs using hybrid mAbs enhances Ab titer production by greater than 100 fold in the absence of any adjuvant (Corbett et al., 2005). The fusion mAbs used in the past were 100% rat including the replacement of the Ab constant regions with a rat IgG1 Fc portion or rat IgG2a Fc portion (Boscardin et al., 2006; Corbett et al., 2005). Therefore, upon *in vivo* administration, high Ab titers were yielded against the injected rat Ab itself, due to the species mismatch (Corbett et al., 2005). As a result, this study took a new approach to generating mouse fusion mAbs in an attempt to avoid Ab production against the construct itself. The constructs used in this study were reengineered as hybrids of rat and mouse; the constructs are ¾ mouse (comprised of a different Fc portion than used in previous experiments, (Corbett et al., 2005): *mouse* IgG2a) and ¼ rat (consisting of the original rat F(ab), which was maintained to ensure binding to the FIRE/CIRE DC surface receptors). Thus, it is expected that there will be a very low, if any,

antibody response to the mAb construct itself upon injection, but ideally, high Ab titers against the antigen of interest “X” will be produced.

In order to evaluate Ab production, mice will be bled, and if any anti-protein “X” Abs are produced, they will be detected by ELISA. If this mechanism is successful in breaking tolerance, it will be applicable to many other candidate vaccine proteins that are highly conserved between mouse and human and are weakly immunogenic. These fusion proteins can then potentially be humanized and applied to human studies of diseases. Ultimately, this mechanism may serve as new methodology for human drug development and vaccinations.

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