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Immunoglobulins and Immunoglobulin Fc Receptors in Nonhuman Primates Commonly Used in Biomedical Research

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**IMMUNOGLOBULINS AND IMMUNOGLOBULIN FC RECEPTORS IN
NONHUMAN PRIMATES COMMONLY USED IN BIOMEDICAL RESEARCH**

by

KENNETH ALTON ROGERS

Under the Direction of Roberta Attanasio

ABSTRACT

Antibodies neutralize and eliminate pathogens, malignancies, and toxins by acting either alone or in association with Fc receptors which, once engaged, activate the elimination mechanisms of phagocytic cells. Based on structural differences, antibodies are divided into functionally distinct classes (IgM, IgD, IgG, IgE and IgA). Structure-function relationships within these classes are not well characterized. In addition, animal models for the assessment of potential therapeutic strategies for the modulation of the interaction between antibodies and Fc receptors are not established. Nonhuman primates are widely used to model human diseases and, represent excellent *in vivo* systems for this assessment. Therefore, we have studied nonhuman primate IgD as well as IgG and IgA specific Fc receptors in rhesus macaques, cynomolgus macaques, baboons and sooty mangabeys. IgD genes had not been identified in nonhuman primates nor the IgD receptors characterized in any species. We characterized IgD genes of the four monkey species, as well as chimpanzees and dogs. In contrast to other antibody classes, the IgD hinge regions are highly conserved between human and nonhuman primates, thus indicating a role in Fc receptor binding. In humans, Fc receptors CD16a (natural killer

cells) and CD16b (neutrophils) bind IgG1 and IgG3, and CD89 (myeloid cells) binds IgA. To assess ligand binding and glycosylation properties of nonhuman primate CD16a, CD16b, and CD89, we sequenced, cloned, and generated recombinant molecules in a mammalian expression system. Our results verify the presence of CD16a, but not CD16b in nonhuman primates. CD16a is expressed on monocytes and a subpopulation of lymphocytes. In sooty mangabeys, CD16 is also expressed on neutrophils. Recombinant sooty mangabey/baboon CD16a binds to human IgG1 and IgG2, but not IgG3 and IgG4. Monkey CD89 has the same peripheral blood leukocyte expression profiles as humans, and binds human and recombinant macaque IgA. Blocking of N-glycans inhibited expression of CD89, but only marginally CD16a expression. Although extensive similarities of antibody/Fc receptor interactions exist between human and nonhuman primates, several differences must be considered when evaluating therapeutic strategies. However, these differences can be exploited to further characterize the structure-function relationships existing within antibody molecules and respective receptors.

INDEX WORDS: Immunoglobulin, Immunoglobulin Fc receptor, Nonhuman primates, Therapeutic antibodies, Animal models, CD16, CD89

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KENNETH ALTON ROGERS

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2006

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KENNETH ALTON ROGERS

Major Professor: Roberta Attanasio
Committee: Phang C. Tai
Barbara Baumstark
Franco Scinicariello

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
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LIST OF ABBREVIATION

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
CART	Conserved antigen receptor transmembrane motif
CD	Cluster of differentiation
CH	Heavy chain constant domain
CL	Light chain constant domain
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EC	Extracellular domain
ELISA	Enzyme linked immunosorbent assay
Fab	Antigen binding fragment
Fc	Fragment crystallizable
Fc α RI	Immunoglobulin A Fc receptor I
Fc ϵ RI	Immunoglobulin E Fc receptor I
FcR	Immunoglobulin Fc receptor
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidylinositol

HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgAN	Immunoglobulin A nephropathy
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
<i>IGHA</i>	Immunoglobulin heavy constant alpha gene
<i>IGHD</i>	Immunoglobulin heavy constant delta gene
<i>IGHG</i>	Immunoglobulin heavy constant gamma gene
<i>IGHM</i>	Immunoglobulin heavy constant mu gene
IgM	Immunoglobulin M
ILR	Immunoglobulin-like receptor
ITAM	Immunoreceptor tyrosine activation motif
ITIM	Immunoreceptor tyrosine inhibition motif
KIR	Killer cell immunoglobulin-like receptor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MYA	Million years ago
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phycoerythrin
pIgR	Poly-immunoglobulin receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TH	T helper

CHAPTER 1

Introduction and Study Objectives

Organisms as diverse as bacteria and humans have evolved defense mechanisms against pathogens. In simple organisms, defenses are generally limited to the level of single cells. For example, bacteria produce endonucleases to cleave DNA of invading bacteriophages as they enter the cell (King and Murray, 1994). With the increasing complexity of the organism, the task of defense becomes more intricate. Complex multicellular organisms must not only mount a defense at the level of the single cell, but must coordinate a defense of the organism as a whole, while preventing any detrimental responses. The immunity of more complex organisms is distinguished by the development of a proper immune system consisting of specialized cells and tissues dedicated to controlling pathogens, toxins and malignancies. In the lower organisms these defenses are limited to innate immunity, which is established prior to the encounter with a pathogen and does not require education of the immune system to function (Akira *et al.*, 2006).

Innate immunity has been highly conserved throughout evolution, and entails specialized cells and molecules that recognize and target foreign structural components common to different classes of pathogens. For example, toll-like receptors are molecules expressed by cells to detect pathogen components, such as bacterial cell wall molecules and DNA, and are found in species from insects to vertebrates (Akira *et al.*, 2006).

Specialized cells of the innate immune system include several types of phagocytic cells: neutrophils, eosinophils, monocytes, macrophages, and dendritic cells (Kuby, 1997). These cells engulf and inactivate pathogens and substances that need to be cleared from the body. Toll-like receptors and other receptors on the surface of phagocytes may bind directly to substances to initiate phagocytosis, or the substances may first be coated with serum proteins called opsonins that are recognized by phagocytes and assist in phagocytosis (Henricks *et al.*, 1986). Opsonins include complement proteins of the innate immune system and antibodies, also called immunoglobulins, of the adaptive immune system (Rus *et al.*, 2005; Henricks *et al.*, 1986). Other cells of the innate immune system, including basophils, mast cells and natural killer cells, are not phagocytic and use other mechanisms to kill pathogens (Kuby, 1997). For example, natural killer cells recognize infected and malignant cells, and can kill these cells by signaling the cells to undergo apoptosis (O'Connor *et al.*, 2006).

Although innate immunity is critical for survival, over time pathogens have evolved mechanisms to evade and overcome innate immunity (Finlay and McFadden, 2006). It was under this selective pressure that an adaptive immune system evolved in vertebrates. The adaptive immune system first emerged in early fish and consists of lymphocytes and their products, which likely evolved from the more ancient innate immune system (Cooper and Alder, 2006). As its name suggests, adaptive immunity is not preexistent to an encounter with a specific pathogen as is innate immunity. Instead, adaptive immunity develops with specificity for a pathogen when the body encounters that particular pathogen for the first time. Adaptive immunity is further distinguished

from innate immunity because of a unique property: memory (Nayak *et al.*, 2005). That is, upon subsequent infection with the same pathogen, the adaptive immune system remembers the pathogen and mounts a faster and stronger response to eliminate the pathogen from the body. However, the adaptive immune system does not operate alone, but works in coordination with the innate immune system. The role of antibodies as opsonins directing phagocytosis has already been mentioned. Conversely, innate immune cells play roles in activating the adaptive immune system (Clark and Kupper, 2005).

The cells of adaptive immunity are B and T lymphocytes. Lymphocytes make up 20-40% of the human leukocyte population and also include natural killer cells (Kuby, 1997). B lymphocytes, or B cells, mature in the bone marrow. T lymphocytes, or T cells, mature in the thymus. B cells are responsible for the production of antibodies, which are the major effector molecules of humoral immunity. On the basis of their function in the immune response, T cells are divided into two categories: T helper (TH) lymphocytes and cytotoxic T lymphocytes (CTLs) (Fabbri *et al.*, 2003). TH cells orchestrate the activity of other cells of the immune system by releasing messenger molecules known as cytokines and are subdivided into T helper type 1 (TH1) and T helper type 2 (TH2) cells on the basis of the cytokines they produce. TH1 cytokines preferentially direct immune responses against pathogens that invade cells and against tumors, whereas TH2 cytokines preferentially direct immune responses against extracellular pathogens (Mosmann and Sad, 1996). CTLs eliminate target cells, such as tumor cells or cells infected by viruses, by producing molecules that form pores on their surface. CTLs then use the pores on the

target to insert additional molecules that specifically induce cell death (Andersen *et al.*, 2006).

The keystones to adaptive immunity are receptors that recognize parts of the pathogens referred to as antigens. These receptors are of two different types that are known as B cell receptors (BCR) and T cell receptors (TCR) for the respective lymphocytes on which they are found. BCRs are composed of membrane bound immunoglobulin molecules and ancillary signaling proteins (Ollila and Vihinen, 2005). In contrast to receptors of innate immunity, BCR and TCR expression involves reorganization of the genes encoding them (Ollila and Vihinen, 2005; von Boehmer, 2006). This reorganization occurs in the lymphocyte progenitor cells, found in the bone marrow for B cells and in the thymus for T cells, and involves stochastic shuffling of gene segments and insertion of nucleotides to create receptors with unique antigen specificity that are exclusive to each differentiated lymphocyte and its daughter cells (Ollila and Vihinen, 2005; von Boehmer, 2006). Each clone produced in this manner undergoes negative selection whereby clones with antigen receptors that recognize self-proteins are induced to undergo apoptosis or to enter a state of inactivation known as anergy (Kappler *et al.*, 1987; Ferry *et al.*, 2006). Unfortunately, this process is imperfect and can lead to autoimmune disorders in which the body attacks itself (Copper and Alder, 2006). Hence, autoimmunity is a byproduct of a dysfunctional adaptive immune system. TCRs only recognize processed antigen presented by major histocompatibility molecules (MHC) expressed on the surface of cells, in contrast to BCRs that recognize unprocessed antigen directly (Kuby, 1997). Therefore, T cells also undergo positive selection. The

process of positive selection allows only development of T cells bearing TCRs that recognize MHC molecules (Kersh, 2004). All other T cells undergo apoptosis.

Newly formed B cells and T cells are naïve and must first be activated prior to performing the immune effector functions that ultimately eliminate pathogens (Kuby, 1997). B cell activation generally requires two signals. The first signal is provided when antigen is recognized by the BCR. The second signal comes from the cognate interaction with a T cell. The antigen bound to the BCR is internalized, processed, and presented as a peptide on MHC class II molecules to a T helper cell (McHeyzer-Williams and McHeyzer-Williams, 2005). T helper cells that recognize the antigen through their TCR become activated and provide costimulatory signals and cytokines that complete the activation process of B cells (McHeyzer-Williams and McHeyzer-Williams, 2005). Some antigens provide a strong enough signal to activate B cells in the absence of T cells. These antigens are known as T cell independent antigens, an example of which is vesicular stomatitis virus that has multiple identical antigen binding sites, or epitopes (Freer *et al.*, 1994). By having these epitopes located in close proximity to each other, several BCRs are brought together by binding a single virion at once. This cross-linking of the BCR then leads to an intracellular signaling cascade that activates the B cell. Once activated, B cells proliferate and differentiate into effector memory cells and antibody secreting plasma cells (Shapiro-Shelef and Calame, 2005; McHeyzer-Williams and McHeyzer-Williams, 2005). As mentioned, antigen presented in the context of MHC class II molecules can activate T helper cells. For this reason, B cells along with macrophages and dendritic cells that also bear MHC class II molecules are known as

professional antigen presenting cells (APC). CTL like T helper cells must be presented with antigen in the context of MHC molecules, but CTL recognize antigen presented by MHC class I molecules, which present endogenous antigens and are expressed on virtually all cell types (the only exception is represented by red blood cells) (Kuby, 1997). CTLs activated through recognition of MHC class I presented antigen kill the presenting cell. Adaptive immune responses are classified as humoral or cellular based on whether the response to clear antigen involves antibodies, or cellular mechanisms, such as those used by CTL.

Antibodies play vital roles in immune defenses. Without medical intervention, individuals born with antibody deficiencies succumb to infections and die early in life (Bonilla and Geha, 2006). Even before the adaptive immune system has developed and produced antibodies of its own, fetuses and infants receive protection from maternal antibodies transferred across the placenta (Simister, 2003). At mucosal surfaces, antibodies are important for blocking the initial entry of bacteria and viruses into the body (Woof and Kerr, 2006). If such a barrier is bypassed, then antibodies are employed in systemic defense. In addition to their role in killing pathogens and neutralizing viruses and toxins, antibodies coordinate the immune response by acting as adaptor molecules that bring antigens together with cells and proteins of the innate immune system (Burton, 2002). These interactions are made possible by the unique structure of antibodies.

Immunoglobulin (antibody) structure

Immunoglobulins are composed of four peptides, two identical light chains and two identical heavy chains (Figure 1.1). Each heavy chain is covalently linked to the other by one or more disulfide bonds, and a single light chain is linked to each heavy chain by an additional disulfide bond (Kuby, 1997). At the quaternary level the immunoglobulin is Y-shaped (Ramsland and Farrugia, 2002). Each immunoglobulin chain is divided into a series of immunoglobulin domains that all share a common tertiary immunoglobulin fold held together by an intra-domain disulfide bond. Immunoglobulin domains are further subdivided into variable immunoglobulin domains and constant immunoglobulin domains. A single variable immunoglobulin domain is located at the amino-terminal end of each immunoglobulin chain, followed by one constant domain in the light chains (CL) and three to four constant domains in the heavy chains (CH). Together, one variable domain from a light chain and one from a heavy chain form the antigen-binding portion of the antibody (Kuby, 1997). Since immunoglobulins have two pairs of heavy and light chains, each molecule has two antigen binding sites at the ends of the arms of the Y-shaped molecule, making it divalent. Variable domains vary extensively in their amino acid content as a result of the previously mentioned reorganization of the gene segments that encode them (Fugmann *et al.*, 2000). It is this diversity of the variable domains that allows antibodies of each B cell and its clones to have unique antigen binding properties. By contrast, constant domains are highly conserved and form the structure of the immunoglobulin that allows it to interact with other components of the immune system to elicit immune effector functions that destroy

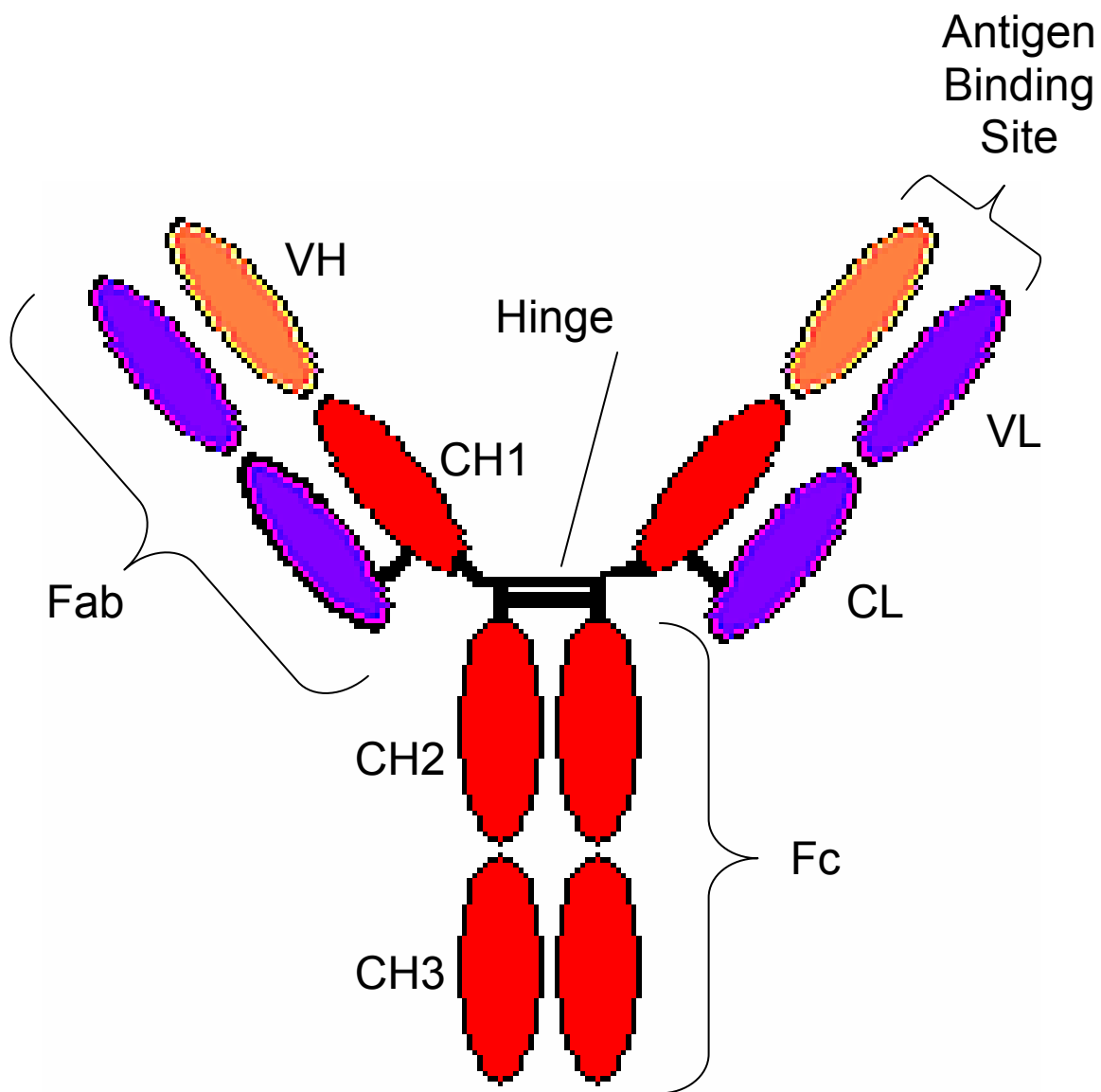


Figure 1.1. Structure of an immunoglobulin molecule. Immunoglobulin light chains consisting of VL and CL domains are shown in blue. Immunoglobulin heavy chains are shown in orange and red and consist of VH, CH1, CH2, and CH3 (some have a CH4). The four polypeptide chains are linked together by disulfide bonds (Kuby, 1997). VL: light chain variable domain; VH: heavy chain variable domain; CL: light chain constant domain; CH heavy chain constant domain.

and eliminate immunoglobulin-tagged antigens (Kuby, 1997).

The original elucidation of the structure of immunoglobulin molecules was aided in the 1950's and 1960's by experiments carried out by Rodney Porter. In these experiments, immunoglobulins of the IgG class were proteolytically fragmented (Porter, 1967). Since the resulting fragments of the immunoglobulin are functionally different, it is still convenient to refer to the portions of an immunoglobulin by the names of these fragments. Digestion with pepsin cleaves immunoglobulins at the heavy chains between the first and second constant domains in a region known as the hinge. This releases two fragments, the $F(ab')_2$, which is capable of binding antigen and the F_c , so named because the fragment is easily crystallized. Alternatively, papain cleaves also at the hinge, but at a position N-terminal to all the disulfide bonds that hold the heavy chains together. Therefore papain digestion releases a similar F_c fragment and two antigen binding Fab fragments. Primarily, it is through the F_c portion that other immune system components bind immunoglobulins to initiate immune effector functions. For this reason, immunoglobulin receptors that bind this portion the immunoglobulin are called F_c receptors (F_cR) (Ravetch and Kinet, 1991).

The constant domains of each immunoglobulin peptide are encoded by constant region genes located downstream of the variable region genes, which recombine together to encode the variable domain of the same immunoglobulin chain. In humans, constant region genes are located at three loci. Immunoglobulin light chain constant regions are encoded by either the κ light-chain locus on chromosome 2 or by the λ light-chain locus on chromosome 22 (Lefranc and Lefranc, 2001). Accordingly, the resulting light chains

are referred to as κ and λ light. The heavy chain constant region locus on chromosome 14 has nine functional heavy chain constant region genes and three pseudogenes. The nine heavy chain regions that are encoded by the functional genes are grouped into five types: μ , γ , α , δ , and ϵ , which in turn define the five isotypes or classes of immunoglobulins: IgM, IgG, IgA, IgD and IgE respectively (Lefranc and Lefranc, 2001). IgG antibodies can be further subdivided into four subclasses (IgG1, IgG2, IgG3 and IgG4), whereas IgA antibodies are found as two subclasses (IgA1 and IgA2). The heavy chains of the antibody subclasses are designated $\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$ (for IgG1, 2, 3 and 4) and $\alpha 1$, $\alpha 2$ (for IgA1 and IgA2). The constant region heavy chain genes are named according to the standardized ImmunoGeneTics nomenclature as *IGH*, followed by the appropriate letter and number to designate the specific subclass, i.e. *IGHG3* for the $\gamma 3$ gene, and *IGHA1* for the $\alpha 1$ gene (Lefranc and Lefranc, 2001). Immunoglobulins of a common class have highly conserved peptide sequences, share common features and have similar functional properties. The immunoglobulin classes IgA, IgD and IgG have heavy chains containing three immunoglobulin constant domains with a hinge that separates the CH2 and CH3 domains. IgM and IgE by contrast have four CH domains and no hinge. Most of the differences between immunoglobulin subclasses are located in the hinge region.

The hinge region contributes to segmental flexibility (Nezlin, 1990) and in IgA and IgG also to intermolecular covalent assembly (Coloma *et al.* 1997). Variability of the length and amino acid content of the hinge region from each subclass results in a different degree of flexibility and to the overall shape of the immunoglobulin (Løset *et al.*, 2005; Sun *et al.*, 2005). The hinge of IgG consists of three segments, the upper,

middle and lower hinge. The middle hinge is a rigid region that contains a variable number of cysteines, which form the inter-chain disulfide bonds by connecting two parallel polyproline double helices. It is connected to the Fc region by the lower hinge and to the Fab portion by the upper hinge. The lower hinge is involved in binding the low affinity Fc gamma receptors (Radaev and Sun, 2001). In humans, the *IGHG1*, *IGHG2* and *IGHG4* genes include only one separate hinge exon, whereas the *IGHG3* hinge is usually encoded by four exons and less frequently by two, three, or five distinct exons (Dard *et al.*, 1997; Lefranc and Lefranc, 2001). Since the IgG3 hinge region is encoded by more than one exon, IgG3 molecules are characterized by hinge regions exhibiting several repetitions and are therefore longer than the hinge regions of the other IgG subclasses. Consequently, IgG3 molecules are the most flexible of all human IgG subclasses (Roux *et al.*, 1997). IgA1 molecules include an elongated hinge region rich in proline residues. The IgA2 hinge region differs from the IgA1 hinge region by a 13-residue stretch of amino acids, presumably the result of an evolutionary response to bacterial IgA1 specific proteases (Kerr, 1990). The human IgD hinge region includes 64 amino acid residues and is, therefore, longer than the hinge region of the other antibody classes (with the exception of IgG3). Hinge regions are characterized by evolutionary instability, as they are the most diverse at the interspecies and intraspecies level (Flanagan, 1984; Scinicariello and Attanasio, 2001; Attanasio *et al.*, 2002; Sumiyama *et al.*, 2002; Scinicariello *et al.*, 2004). Differences in the hinge of each molecule have been demonstrated to alter immunoglobulin affinity for antigen and immune complex formation (Roux *et al.*, 1997; Løset *et al.*, 2004). In some HIV *in vitro* studies IgG3 with

its long hinge has been demonstrated to be more efficient than other IgG subclasses at neutralizing viruses in the absence of affinity differences (Cavacini, 1995; Scharf *et al.*, 2001). Hence the hinge region greatly contributes to antigen binding and antigen elimination.

Immunoglobulins are expressed as BCR or as secreted proteins based on the type of carboxyl-terminal tail they express. Immunoglobulins forming BCRs are anchored to the plasma membrane by a hydrophobic tail that makes up the transmembrane domain and a short cytoplasmic tail. The transmembrane domains of all antigen receptors, including TCRs, have residues that make up a conserved antigen receptor transmembrane motif (CART). This motif is believed to be necessary for proper association with the signaling molecules of the antigen receptor complex (Campbell *et al.*, 1995). For BCR, these signaling molecules are the invariant transmembrane proteins Ig- α and Ig- β , which contain the necessary cytoplasmic elements for signaling and antigen presentation (Cambier *et al.*, 1994). For all human immunoglobulin heavy chains, the immunoglobulin transmembrane tail is encoded by two exons (M1 and M2) found 3' to the last CH exon of the gene, whereas secreted antibodies have a short hydrophilic tail that varies greatly between the different immunoglobulin isotypes. For all isotypes except IgD, the secretory tail is encoded at the 3' end of the final CH exon. The IgD secretory tail has been found to be encoded by a separate exon (CH-S) located between the CH3 exon and the exons M1 and M2 of the Ig δ gene (*IGHD*) in all species in which it has been characterized. Polyadenylation of polyA motifs found 3' of the tail to be used and subsequent alternative splicing regulate production of the cytoplasmic tail.

Immunoglobulin subclasses and functions

Antibodies alone in some cases are sufficient to inactivate a pathogen to prevent infection or render toxins innocuous. These antibodies are called neutralizing antibodies. The mechanisms responsible for neutralization are not fully understood (Burton, 2002). Neutralizing antibodies may defend against viruses by occupying all possible virion sites of interaction with host cells, thus blocking infection. Evidence indicates that other antibody properties are usually required to prevent or stop infections. Fab fragments from neutralizing antibodies generally lose the ability to prevent disease (Burton, 2002). Indeed, many bacteria actively produce proteases that separate antigen binding Fab fragment from the Fc fragment of the antibody (Kilian *et al.*, 1996). It has been suggested that some gut bacteria may be able to use IgA Fab fragments created by their proteases to cloak antigen epitopes from other intact antibodies to escape targeting by the immune system. Other bacterial proteins like staphylococcal superantigen-like protein 7 bind the Fc region of IgA antibodies, thereby blocking interactions with complement and Fc receptors (Langley *et al.*, 2005).

Each immunoglobulin subclass has different properties and consequently performs different functional roles in the immune system (Figure 1.2 and Table 1.1) (Spiegelberg, 1989). Particularly, immunoglobulin functional differences include eliciting different immune effector mechanisms (complement-dependent cytotoxicity, antibody-

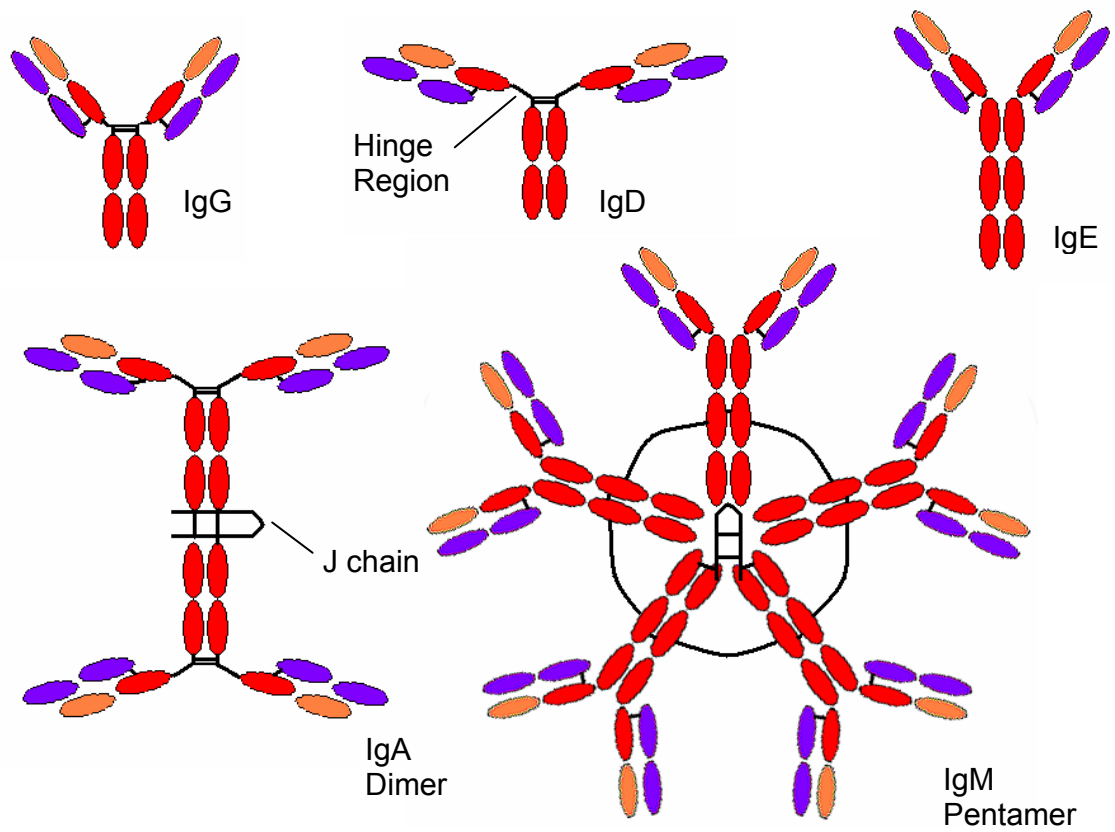


Figure 1.2. Structure of the five mammalian classes of immunoglobulins. Diagram is an adaptation from Kuby (1997).

Table 1.1. Functional properties of the human immunoglobulin subclasses (Kuby, 1997).

Functional Property	Immunoglobulin								
	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Complement Activation (Classic pathway)	++	+	+++	-	+++	-	-	-	-
Complement Activation (Alternative pathway)	-	-	-	-	-	+	-	+	-
Placental transfer	+++	+	++	-/+	-	-	-	-	-
Binding to phagocyte Fc receptors	+	-	+	-/+	-	+	+	-	+
High-affinity binding to mast cells & basophils	-	-	-	-	-	-	-	-	+++
Neutralization	++	++	++	++	+	++	++	-	-
Opsonization	+++		++	+	+	+	+	-	-
Sensitization for killing by NK cells	++	-	++	-	-	-	-	-	-
Sensitization of mast cells	-	-	+	-	-	-	-	-	+++

dependent cellular cytotoxicity, and opsonization), physiological localization, ability to multimerize, ability to cross the placenta, and secretion at mucosal surfaces (Burton and Woof, 1992; Jefferis *et al.*, 1998). Each subclass within an immunoglobulin class appears similar to the other (only a 5-10% gene difference for the IgG subclasses). However, there are important differences between them. Production of a specific subclass is controlled by genetic rearrangement at the heavy chain gene locus, a process called isotype switching (Stavnezer, 1996). This process results in a heavy chain consisting of the same variable domain (and therefore with identical antigen specificity). The environmental milieu of a B cell dictates which subclass of antibody is produced and is a reflection of the assault with which the immune system is challenged. For example, a viral infection stimulates T helper type I cytokines that will result in isotype switching to produce antibody classes best able to fight intracellular infections.

IgM and IgD with the same antigen specificity are coexpressed on the surface of naïve B cells and act as the initial BCR to bind antigen. No other combination of two immunoglobulins can be expressed. Uniquely, Ig μ and Ig δ genes are transcribed in a single RNA molecule which is then polyadenylated 3' of either the Ig μ exons or the Ig δ exons and then spliced (Preud'homme *et al.*, 2000). Of the two, IgD is the least understood. Results from a few recent studies show that IgD and IgM may modulate B cell activation differently. Secreted IgD makes up only about 0.5% of serum immunoglobulins and very little research has been performed to determine its role (Brandzaeg *et al.*, 1991). Both membrane and secreted IgD can bind to receptors expressed on T cells, hence modulating immune responses (Amin *et al.*, 1991; Rudd *et*

al., 1995). IgM constitutes 5-10% of the total serum immunoglobulin. Low affinity IgM is the first immunoglobulin secreted in a primary immune response and is thought to represent the major isotype of natural antibodies. Natural antibodies are secreted in the absence of prior exposure to antigen, and often have broad antigen specificity. Natural antibodies may play an important role by priming the immune response through an initial capture of pathogens for processing by APC (Ochsenbein *et al.*, 1999). Multimeric IgM is efficient at activating the classical complement pathway. IgD does not form multimers and does not activate the classical complement pathway.

Both IgA and IgM multimerize and are transported across epithelial cells to mucosal surfaces (Brandzaeg *et al.*, 1999). IgM molecules form pentamers and IgA predominantly forms dimers, although IgA polymers can exist also in trimeric and tetrameric forms. Multimerization is facilitated by the attachment of a polypeptide chain called the J chain. The J chain in turn can associate with the poly-immunoglobulin receptor (pIgR) expressed on the basolateral surface of epithelial cells. The pIgR then endocytoses and transports the immunoglobulin to the apical surface. The pIgR is then cleaved to release the immunoglobulin, leaving a portion of the pIgR (referred to as the secretory component) still attached to the molecule. IgA is the most abundant immunoglobulin class, accounting for 10-15% of serum immunoglobulin and for almost all of secreted immunoglobulin (76-90% in the gut mucosae, 69-86% in nasal, lacrimal and parotid glands) (Brandtzaeg *et al.*, 1999). In the mucosae, IgA acts via immune exclusion by coating pathogens and preventing them from binding to the mucosal linings. IgA also helps with clustering of pathogens and their subsequent expulsion from the

mucosae (van Egmond *et al.*, 2001). IgA is considered an anti-inflammatory immunoglobulin. Through binding Fc receptors (Fc α RI or CD89), IgA can down regulate the effector functions of leukocytes from the myeloid lineage, such as inflammatory cytokine release induced by IgG immune complexes. Somewhat paradoxically, IgA immune complexes can interact with the same receptor to induce leukocyte effector functions (Pasquier *et al.*, 2005).

IgG makes up 80% of serum immunoglobulins with the relative abundance of the human subclasses being IgG1>IgG2>IgG3>IgG4 (Kuby, 1997). IgG are usually high affinity antibodies and are produced late in a primary immune response. IgG modes of action differ between subclasses. IgG3 followed by IgG1 then IgG2 can activate complement, whereas IgG4 cannot. IgG1, IgG3 and IgG4 are transported across the placenta to protect the fetus. IgG1 and IgG3 are most efficient at binding Fc receptors (Gessner *et al.*, 1998).

IgE has a very low serum concentration and is mostly bound to mast cells and eosinophils through the high affinity IgE Fc receptor. Antigen cross-linking of Fc receptor bound IgE on these cells results in the release of type I hypersensitivity reactions mediators. Type I hypersensitivity reactions range from common allergies to anaphylaxis. In addition, IgE plays an important role in defense against parasites (Kuby, 1997).

Immunoglobulin Fc receptors

The humoral immune system consists mostly of antibodies and is connected to the cellular immune system through immunoglobulin Fc receptors (FcRs). FcRs exist for all antibody isotypes and are present on all leukocytes (Ravetch and Kinet, 1991). However, different Fc receptors are present for the different types of leukocytes and each is specific for one or a few immunoglobulin subclasses. Antigen/immunoglobulin complexes act to cross-link FcR and subsequently set off intracellular signaling cascades. FcRs contain either immunoreceptor tyrosine activation motifs (ITAMs) or immunoreceptor tyrosine inhibition motifs (ITIMs), which function to activate or inactivate cells, respectively. The first step of the signaling cascades is the phosphorylation at tyrosines of one of these motifs (Cox and Greenberg, 2001). Most leukocytes have both ITAM and ITIM-containing receptors and the balance of these competing signals determines the activation status of the cell. Although FcR activation is similar in different classes of leukocytes, the induced effector functions are dictated by which type of leukocyte is activated. FcR-induced effector functions that directly target antigens include phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and respiratory burst (Ravetch and Kinet, 1991). Additionally, FcR-controlled events include release of cytokines and inflammatory mediators, and antigen presentation. The IgA Fc receptor I, CD89, can regulate IgA half-life by protecting endocytosed IgA for transport back out of the cell or by transporting it to the lysosome compartment for degradation (Launay *et al.*, 1999). IgG Fc receptors may modulate antibody production by IgG-mediated feedback suppression or by enhancement of antibody responses (Heyman, 2003).

FcRs are diverse in their structure, but share common features and nomenclature. FcRs were first described in the 1970's, and the IgE high affinity immunoglobulin receptor (FcεRI) was the first characterized at the molecular level. FcεRI is made up of four polypeptide chains: α , β , and a γ dimer formed by an inter-chain disulfide bond (Conner and Saini, 2005). The FcεRI α chain is the IgE ligand binding chain. The β and γ chains are responsible for intracellular signaling and each consists of a transmembrane portion and a cytoplasmic tail containing ITAMs. Subsequent to the description of FcεRI, other FcR (FcγRI, FcγRIII and FcαRI) were found to consist also of unique ligand binding α chains and the same γ chain dimer (FcR γ_2) (Ravetch and Kinet, 1991). The α chains associate with FcR γ_2 through conserved charged residues in the transmembrane domain of each chain. Alternatively, in human natural killer cells FcR γ_2 can be substituted with a TCR ζ signaling chain dimer or a heterodimer of a TCR ζ and FcR γ (Lanier *et al.*, 1989). For the IgG receptors FcγRI and FcγRIIIa, the signaling chains have been shown to be required for efficient transport from the endoplasmic reticulum to the plasma membrane, whereas the IgA receptor FcαRI may be transported to the plasma membrane without association with FcR γ_2 (van Egmond *et al.*, 2001). In contrast to other receptors, the IgG Fc receptors FcγRIIa and FcγRIIb consists of single α chain only with an extended cytoplasmic tail that contains either an ITAM or ITIM, respectively (Ravetch and Bolland, 2001). Human neutrophils may also express FcγRIIb. This receptor is almost identical to FcγRIIIa, but is truncated and connected to the plasma membrane by a GPI-link. This receptor lacks any signaling motif and functions by modulating the function of other neutrophil FcRs with which it becomes cross-linked (Ravetch and Bolland, 1998). FcR α chains are members of the immunoglobulin superfamily,

because they all have two or more immunoglobulin-like domains forming their extracellular portion. The IgG Fc receptors bind IgG with their membrane proximal immunoglobulin-like domain at an 1:1 ratio (Radaev and Sun, 2001). By contrast, Fc α RI binds IgA at its first immunoglobulin-like domain in a 2:1 ratio (Herr *et al.*, 2003). FcRs have different affinities for immunoglobulins. The high affinity Fc ϵ RI allows it to bind to monomeric IgE, thus resulting in a low concentration of free IgE in serum (Conner and Saini, 2005). Fc receptors with low affinities usually bind antibodies as part of immune complexes.

Fc receptors are important not only for initiating immune responses to pathogen. IgG receptors are polymorphic within the human population and it is now well established that certain polymorphisms are risk factors for susceptibility to some infectious diseases and autoimmune disorders (van der Pol and van de Winkel, 1998). Polymorphisms of Fc γ RIII are associated with severity of Guillain-Barré syndrome (Sorge *et al.*, 2005). Soluble Fc α RI released from cells has been proposed in a model for the mechanism of IgA nephropathy, the most common form of primary glomerulonephritis and renal failure (Launay *et al.*, 1999; Monteiro *et al.*, 2002; Monteiro, 2005). Although this model is still hotly debated, there is a tacit consensus that one or more Fc receptors are likely to be involved in IgA nephropathy (van der Boog, 2003; van der Boog *et al.*, 2004). Fc receptor interactions are also critical to the design and use of therapeutic antibodies. Monoclonal antibodies are effectively used to prevent and/or control a variety of disorders, including cancer, autoimmune diseases and infectious diseases (Schulze-Koops and Lipsky, 2000; Zeitlin *et al.*, 2000; Park and Smolen, 2001). At least eleven therapeutic antibodies are currently approved for human therapy and the need for novel engineered antibodies continues to expand (Presta, 2002; Cohen-Solal,

2004). Currently, over 300 different clinical trials involving monoclonal antibodies are listed on the National Institute of Health website (<http://www.clinicaltrial.gov>). A clear understanding of the mode of action of monoclonal antibodies should take into account Fc receptor interactions. Antibodies designed to ameliorate autoimmune diseases often have as their goal to stimulate inhibitory receptors like Fc γ RIIb, while at the same time avoiding stimulation of activating receptors. For antibodies being used for cancer treatment just the opposite is true. Therefore, antibodies with modified Fc regions are being engineered. Such modifications can be designed only if a detailed characterization of Fc receptor/antibody interaction is available (Newman *et al.*, 2001; Bisikirsha *et al.*, 2005). To further complicate matters, engineered bispecific antibodies that have one variable region with specificity for a tumor antigen and the other with specificity for an Fc receptor are considered to have great potential as cancer therapeutics. Even once a monoclonal antibody is developed and shows efficacy in clinical trials, the reasons for the success are often unknown and required further research (Bisikirsha *et al.*, 2005).

Antibodies and their Fc receptors are studied widely in different species to understand their structure and function. It is hoped that information from these studies will help to devise means to better bolster positive or attenuate harmful immune responses in humans and in economically important species. These studies also have yielded important insights into the evolution of the immune system, which has given us a better understanding of host-pathogen interactions. The antibodies and Fc receptors most extensively studied have been those of humans and species important as models of human diseases and immunity. In particular, mice are well studied and have given us great insight into how the immune system operates.

However, mice differ from humans in their humoral immune system. For example, mice have only one subclass of IgA and their IgD is quite structurally distinct from the human IgD (Mushinski *et al.*, 1980; Shimizu *et al.*, 1982). Fc receptors show major differences between mice and humans. Mice have no homologues of several human Fc receptors: IgA Fc receptor I (CD89), IgG Fc receptor IIa (CD32a), and IgG Fc receptor IIIb (CD16b) (Ravetch and Kinet, 1991). Additionally, the mouse IgG Fc receptor CD16a does not appear to be a true orthologue of human CD16a (Hughes, 1996). Indeed, comparative studies with other species show that the biology of antibodies and their receptors is quite diverse throughout vertebrates. Therefore, to perform research with the goal of understanding human humoral immune responses, it is often necessary to use species phylogenetically closely related to humans. Hence, nonhuman primates are often the animal models of choice. This choice is based on the assumption that antibodies and Fc receptors are highly conserved between humans and nonhuman primates. Existing data generally support this assumption, but antibodies and Fc receptors have not been fully characterized in any species including nonhuman primates.

Animal models

The use of animal models in biomedical research has provided and will continue to provide the basis for numerous advances in medicine. Despite ever mounting bioethical pressures to minimize the numbers of animals used in research and despite the increasing availability of *in vitro* experimental systems, animal models are indispensable to understand human physiology and human diseases. Only *in vivo* models can reproduce

the complexity of the interactions connecting the human organ systems. These systems cannot be fully understood through reductive studies of their individual components.

According to the United States Department of Agriculture, an estimated 1.13 million animals were used in research in 2002. Rodents make up the majority of these animals, whereas nonhuman primates account for about one percent (~50,000) of animals used in research. The absolute number of nonhuman primates currently in use has been steady for over 20 years. Recent developments in biomedical research have increased demand for these species, despite considerable efforts made to minimize their use whenever possible. Nonhuman primates remain an appropriate model for research in hematology, immunology and virology (Hérodin *et al.*, 2005; Patterson and Carrion 2004). Furthermore, the demand for nonhuman primates is expected to increase because of needs related to the development of therapeutics and vaccines against potential bioterrorism threats (Patterson and Carrion, 2004). Indeed, the US Food and Drug Administration's new guideline for the assessment of reagents against select agents would require testing in at least two animal models, one of which should be a nonhuman primate (Patterson and Carrion, 2004). The evaluation of therapeutic strategies now includes the understanding of their mechanism of action as well as the careful analysis of potential side effects, thus requiring the presence of minimal differences resulting from species divergence.

Nonhuman primates used in biomedical research are represented by many species. Therefore, we will briefly discuss the evolution of primates and their phylogenetic relationships.

Primate evolution

Although mammals are as ancient as the dinosaurs, the fossil record suggests that mammal diversity was limited until the end of the Cretaceous period, when the die off of the dinosaur allowed for the rapid adaptive radiation of mammals to fill the opening niches. Placental mammals emerged about 140 million years ago (MYA) and radiated during the Cenozoic period. Most of the major groups were established during the Paleocene epoch about 65 MYA. The fossil record indicates that distinct primate forms emerged at this time starting with the prosimians. Only recently some consensus has been reached by taxonomists on the overall relationship of placental mammals. Currently, placental mammals are divided into Afrotheria, Xenarthra, Laurasiatheria, and Euarchontoglires, the group in which primates and rodents are placed (Springer *et al.*, 2004). This new phylogeny clearly removes chiroptera from its previous grouping with primates making flying lemur and members of scandentia the closest relatives of primates. It is also now accepted that rodents are more closely related to primates than to carnivores and ungulates (Springer *et al.*, 2004). Primate evolution is believed to have started in the old world where the ancestors of modern prosimians, Old World monkeys (Catarrhines) and New World monkeys (Platyrrhines), coexisted perhaps up to the late Eocene epoch.

The history of anthropoids (Old World monkeys, New World Monkey and hominoids) is based on the exchange of species between Eurasia and Africa, and it's still

an area of controversy. The latest fossil evidence suggests that the earliest African anthropoids emigrated from Asia before the late Paleocene, just before 37 MYA. At the same time, other species were being swapped between these continents (Jaeger and Marivaux, 2005; Seiffert et al., 2005; Beard 1995). Recently, molecular studies have placed the split off of the New World monkey around 35 MYA. At that time, the ancestors of these monkeys may have crossed the Atlantic to South America where their descendants are found today (Schrago and Russo, 2003). The ancestors of modern Old World monkeys and hominoids, which include humans and the apes, probably stayed in Africa until they split around 25 MYA ago. Further splits occurred about 18 MYA when the lesser apes (Gibbons and Siamangs) split from the great apes, and around 14 MYA when the papionins split from the colobines (Stewart and Disotell, 1998). Investigators generally agree on the relationship of the greater apes: humans are most closely related to chimpanzees, with whom they share a common ancestor about some 5 MYA (Schrago and Russo, 2003; Stewart and Disotell, 1998). By contrast, the relationships between Old World monkey species (papionins, which include baboons, mangabeys, and macaques) are far from resolved. As a general rule, however, the African species of baboons, mangabeys, and mandrill are grouped together apart from the mostly Asian macaques (Stewart and Disotell, 1998). There is only one non-Asian macaque species, *Macaca sylvanus*. This species raises doubts on the monophyletic origin of the genus *Macaca* (Groves, 2000). Figure 1.3 depicts the phylogentic tree of Old World monkeys and hominoids along with a chronology of evolutionary events.

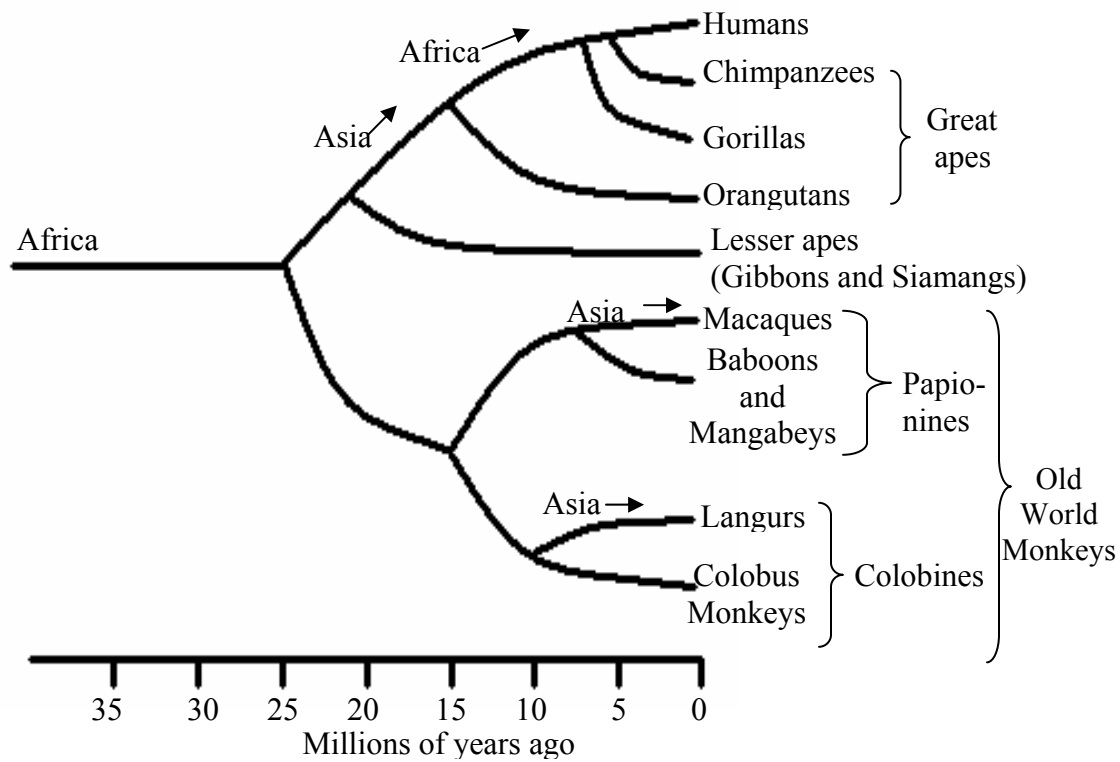


Figure 1.3. Phylogeny of catarrhine primates. Adapted from evolution and phylogeny proposed by Stewart and Distell (1998). Estimated times of splits for the major groups are shown. The intercontinental dispersal events between Africa and Eurasia are indicated by the arrows with the stem of the tree starting in Africa.

Nonhuman primates in research

Evolutionarily humans are most closely related to the great apes (chimpanzees, gorillas, orangutans and gibbons), and therefore our physiology is most similar that of these species (Rogers and VandeBerg, 1998). Only a few studies are carried out in great apes since, they are endangered. In addition, the costs associated with research in these animals are prohibitively expensive (Shearer, 1999). Therefore, most nonhuman primate research is performed using the papionini group of Old world monkeys. A review of publications in which nonhuman primates were recently used shows that the most widely used species are, in descending order, *Macaca mulatta* (rhesus macaque), *Macaca fascicularis* (cynomolgus macaque) and *Papio* spp. (baboons). *Chlorocebus aethiops* (African green monkey), is the most commonly used species when counting *in vitro* studies using cells lines, i.e. Vero cells, which are derived from this species (Carlsson *et al.*, 2004). On a smaller scale, some species of New World monkeys and prosimians are also used in research. The primary areas of research that use nonhuman primates are microbiology including HIV/AIDS (26%), neuroscience (19%), biochemistry (12%) and pharmacology/physiology (11%) (Carlsson *et al.*, 2004).

Macaques

The macaques are included in the living cercopithecine. Macaques used in research include rhesus macaques (*M. mulata*), crab eating or cynomolgus macaques (*M. fascicularis*) and pig-tailed macaques (*M. nemestrina*).

Rhesus monkeys have been the primary primate used in medical research up until recently, but their use is now likely to decrease. Once abundant for use and inexpensive, a decline in their numbers resulted in an embargo in their trade from India in 1978 and subsequently a shift to imports from China which has been accompanied by a rising cost. The vital role of rhesus macaques in HIV pathogenesis and in AIDS vaccine development research has placed this species at the forefront of immunological studies in primates (Earl *et al.*, 2002; Horton *et al.*, 2002; Barber *et al.*, 2004; Evans *et al.*, 2003; Lifson *et al.*, 2003; Monceaux *et al.*, 2003; Willey *et al.*, 2003; Lena *et al.*, 2002). In addition, these monkeys have been and continue to be employed for immunological characterization and vaccine design for viral, bacterial, and protozoan infections associated with Ebola virus (Geisbert *et al.*, 2002), West Nile/dengue virus (Pletnev *et al.*, 2003), hepatitis E (Purcell *et al.*, 2003), *Mycobacterium tuberculosis*, *Borrelia burgdorferi* (Lyme's disease), *Helicobacter pylori* (Del Giudice, 2001; Langermans *et al.*, 2001; Pachner *et al.*, 2001; Lai *et al.*, 2003), *Leishmania major* (Freidbag *et al.*, 2003) and *Plasmodium* species (Angov *et al.*, 2003).

As rhesus macaques become less available and more costly, researchers have focused on other macaques as animal models including pigtail macaques and cynomolgus macaques. Pigtail macaques are now being extensively used as animal models for AIDS-related studies (Qian *et al.*, 1994; Dale *et al.*, 2002; Herz *et al.*, 2002; Yu Kimata *et al.*, 2002; Ambrose, 2003; Shen *et al.*, 2003). They are being developed as animal models for mucosally transmitted HIV-1 infection using R5-tropic SHIV (simian/human immunodeficiency virus) and characterizing neutralization-resistant

virus variant of SHIV (Stephens *et al.*, 1997; Narayan *et al.*, 1999; Chen *et al.*, 2000). Other research studies using pigtail monkeys include studying the pathogenesis of viral gastroenteritis (Subekti *et al.*, 2002) and identifying immunological markers of aging (Bowden *et al.*, 1994). *Cynomolgus* macaques are being used similarly to other macaques species for pathogenesis and vaccine studies a sampling of which include yellow fever-dengue (Guirakhoo *et al.*, 2004), hepatitis E virus (Li *et al.*, 2004), HIV (Reimann *et al.*, 2005), SARS (Qin *et al.*, 2006), scrub typhus (Chattapadhyay *et al.*, 2005) and *Yersina pestis* (bubonic plague) (Honko *et al.*, 2006). Additionally, macaques have frequently been used to test evaluate therapeutic antibodies and have been used in transplantation studies, two area of research that are not mutually exclusive (Cendales *et al.*, 2005; Cozzi *et al.*, 2005; Gaudreault *et al.*, 2005; Martin *et al.*, 2005; Hering *et al.*, 2006; Kim *et al.*, 2006).

Baboons and sooty mangabeys

Two types of African papionini monkeys used in research are baboons (*Papio* species) and sooty mangabeys. Baboons are used as models for numerous biomedical interests including transplantation (Elster *et al.*, 2001; Hoerbelt and Madsen, 2004), therapeutic antibodies (Wu *et al.*, 2002; Deckmyn *et al.*, 2005), vaccine development, and cardiovascular disease (Kushwaha and McGill, 1998; Holm, 2001), which in recent years has been recognized to involve immune responses. The use of baboons benefits several areas of research because many of these animals from the primate centers in the US are pedigreed (VandeBerg and Williams-Blangero, 1997). Baboons are now

commonly used as animal models for the development of several vaccines, including HIV infection/AIDS (Locher *et al.*, 2001; Wang *et al.*, 2002; Locher *et al.*, 2003) influenza virus (Bot *et al.*, 2001), hepatitis B virus (Watts *et al.*, 1999), *Hemophilus influenza* (Shearer *et al.*, 1997), *Mycobacterium tuberculosis* (Pehler *et al.*, 2000), *Neisseria meningitidis* (Granoff *et al.*, 1997), group B *Streptococcus* (Paoletti *et al.*, 2000), and *Schistosoma mansoni* (Farah and Nyindo, 1996). Sooty mangabeys (*Cercocebus torquatus*) are natural hosts of SIV, but rarely develop AIDS-like pathogenesis as do macaques infected with SIV (Ling *et al.*, 2004). Therefore sooty mangabeys represent useful animal models for evaluation of AIDS pathogenesis and potential vaccines (Villinger, 1996; Kaur *et al.*, 2000; Ansari *et al.*, 2003; Bostick *et al.*, 2003; Silvestri *et al.*, 2003; Veazey *et al.*, 2003, Silvestri, 2005). Mangabeys represent a useful model to study leprosy (Wolf *et al.*, 1985; Gormus *et al.*, 1995).

Study objectives

The immune system is an essential defense without which we would quickly succumb to pathogens and malignancy. Just as the challenges to this defense are many, diverse and complex, so is the makeup of the immune system. Although *in vitro* studies are pivotal to advance our understanding of immune responses, *in vivo* studies are indispensable to complete this understanding. Since the first use of vaccines, medical science has constantly strived to develop new ways to manipulate the immune system to bolster or attenuate it where needed for better health. These developments come through

experimentation, which for ethical considerations and availability of proper controls cannot be done in humans. Therefore, animal models are often used in immunological research. Nonhuman primates are excellent animal models because they are most similar to humans when compared to other species commonly used in biomedical research. In order to optimize the value of research performed in nonhuman primates, it is necessary to understand their physiology. Antibodies and hence their Fc receptors are crucial to the adaptive humoral immune system. These molecules are being actively researched and used as tools to modulate the immune responses in humans, yet their structure and functions are not fully characterized, especially for the nonhuman primate species in which testing of immunotherapeutic strategies is carried out.

Our laboratory seeks to understand how the immune system is shaped by and responds to challenges from pathogens and immunosenescence. We have characterized humoral immune responses generated against antigen challenge or as a result of aging in healthy nonhuman primates. In addition, we have characterized the immunoglobulin variable and constant region gene repertoire as well as antibody gene polymorphisms in these species, focusing on IgA and IgG molecules. The present study was designed to expand the characterization of nonhuman primate humoral responses.

Specifically, our objective was to answer the following questions:

- 1) What is the extent of similarity between human antibody genes and nonhuman primate antibody genes, in particular the IgD heavy chain gene? Are IgD molecules conserved in these species and might they have conserved features that suggest shared function? What

insights can this information bring to understanding structure/function relationships in other antibody isotypes?

2) Do humans and nonhuman primates share the same immunoglobulin Fc receptors and are Fc receptors expressed on the same cell types in humans as in nonhuman primates?

3) How do human and nonhuman primate antibodies interact with nonhuman primate Fc receptors?

Answers to these questions will enhance and guide work performed with nonhuman primates, improving our understanding of the strengths and weaknesses of scientific results obtained using these species. Ultimately, this work will provide a foundation for research that focuses on the development of therapies to prevent and treat infectious diseases and cancers, prevent transplant rejection, and control autoimmune diseases.

CHAPTER 2

Identification and Characterization of Macaque CD89 (IgA Fc Receptor)

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Keywords: CD89, IgA receptor, Macaques, Splice variants, Nonhuman primate models

Summary

The interaction of the IgA molecule with its specific cellular receptor is necessary to trigger a variety of effector functions able to clear IgA-opsonized antigens. The human IgA-specific Fc receptor, Fc α RI or CD89, is expressed on cells of the myeloid lineage. Recently, CD89 homologues have been identified in rats and cattle. Because nonhuman primates represent well established models for a variety of human diseases and for the testing of immunotherapeutic strategies, we cloned and sequenced cDNAs corresponding to the CD89 gene from rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques. Macaque sequences of full-length CD89 consist of five exons of length identical to the corresponding human CD89 exons. The rhesus and cynomolgus macaque derived amino acid sequences are highly homologous to each other (99.3% identity) and exhibit 86.5% and 86.1% identity to the human counterpart, respectively. Transfection of HeLa cells with plasmids containing the cloned macaque cDNAs resulted in the expression of surface

molecules recognized by an anti-human CD89 antibody. Five splice variants were identified in rhesus macaques. Three of the five variants are similar to described human CD89 splice variants, whereas two variants have not been described in humans. Three splice variants were identified in cynomolgus macaques. Of the three variants, one is present also in humans and rhesus macaques, whereas the other two are shared with rhesus macaques but not humans. Similarly to the human CD89, macaque CD89 is expressed on myeloid cells from peripheral blood. The characterization of macaque CD89 represents an essential step in establishing a nonhuman primate model for the testing of immunotherapeutic approaches based on the manipulation of the IgA/CD89 interaction.

Introduction

Fc receptors (FcRs) are expressed on a variety of immune effector cells and, by binding the constant region of antibody molecules, provide an essential link between the humoral and cellular arms of the immune system (Ravetch and Kinet, 1991). Fc α RI (CD89), the Fc receptor specific for IgA molecules, is a transmembrane glycoprotein that belongs to the immunoglobulin gene superfamily (Maliszewski *et al.*, 1990). It is expressed on monocytes, eosinophils, neutrophils and macrophages (Monteiro and van de Winkel, 2003) and its signaling capacity is dependent on the association with the FcR γ -chain subunit (Pfefferkorn and Yeaman, 1994). The human CD89 gene is located within the leukocyte receptor cluster on chromosome 19 (Kremer *et al.*, 1992), appears to exist as a single copy (Maliszewski *et al.*, 1990), spans 12 kb and includes five exons (de Wit

et al., 1995). Exons S1 and S2 encode the leader peptide, EC1 and EC2 each specify an extracellular immunoglobulin-like domain of 103 amino acids, and TM/C codes for the transmembrane domain (19 amino acids) and cytoplasmic tail (41 amino acids) of the receptor.

The CD89/IgA interaction mediates immune effector responses, including antibody-dependent cell-mediated cytotoxicity, phagocytosis and respiratory burst, as well as release of cytokines and inflammatory mediators (Monteiro and van de Winkel, 2003). Therefore, this interaction is necessary to maintain the integrity of the immune responses in both systemic and mucosal compartments. Indeed, IgA is the most abundantly produced antibody isotype and represents a first line of defense at mucosal surfaces. The IgA system differs in the animal species most extensively studied. In humans, there are two IgA subclasses, designated IgA1 and IgA2. Both subclasses bind Fc α RI with a low affinity of approximately $K_a = 10^6 \text{ M}^{-1}$ (Wines *et al.*, 1999). Only one IgA subclass is present in mice (Shimizu *et al.*, 1982), whereas rabbits possess 13 IgA subclasses (Burnett *et al.*, 1989). In rhesus macaques, IgA molecules are characterized by an extremely high level of intraspecies heterogeneity (Scinicariello and Attanasio, 2001; Scinicariello *et al.*, 2004). This IgA heterogeneity appears in several nonhuman primate species (Sumiyama *et al.*, 2002). Interestingly, no Fc α RI homologue has been identified in mice. Recently, Fc α RI homologues have been identified in rats (Maruoka *et al.*, 2004), and cows (Morton *et al.*, 2004). Here, we describe the rhesus and cynomolgus macaque CD89.

Materials and methods

Amplification, Cloning and Sequencing of Macaque CD89

Heparinized blood samples were collected from one healthy rhesus macaque (*Macaca mulatta*) and one healthy cynomolgus macaque (*Macaca fascicularis*). Total RNA was extracted from whole blood using the QIAamp RNA Blood Mini Kit (Qiagen Inc., Valencia, CA), and reverse transcribed into cDNA using oligo d(T)17 primers, followed by primer extension with the AMV reverse transcriptase (Roche Diagnostic Corp., Indianapolis, IN). PCR amplification of the cDNA was performed with Expand High Fidelity polymerase (Roche Diagnostic Corp.). The nucleotide sequences of the PCR primers used to amplify the complete CD89 transcripts have been previously reported by Pleass and co-workers (Pleass *et al.*, 1996). Forward (RP1) and reverse (RP2) primers specific for the human CD89 located at the start codon of the S1 exon and the stop codon of the TM/C exon, respectively, were employed. The PCR conditions used were those described by Pleass and coworkers (Pleass *et al.*, 1996) except that forty cycles of PCR were performed.

An alternate form of CD89, known also as Fc α RIb (which lacks exon TM/C but contains nucleotide sequences present in the intron located between exon EC2 and exon TM/C of variant 1) has been described (van Dijk *et al.*, 1996). There are two Fc α RIb isoforms, known as CD89 transcript variants 9 and 10 (van Dijk *et al.*, 1996). In order to amplify this alternate form, we designed the FCARB2 primer (5'-TCTAGCGAGGAAGTGAAAGCGG-3') located at the 1024-1003 nt of the CD89 transcript variant 9 (GenBank NM_133279) that, used with the RP1 primer, allows

amplification of the complete Fc α R1b transcripts. After reverse transcription of total RNA, the cDNA was amplified with the FCARB2 and RP1 primers using the same conditions described for the RP1 and RP2 primers (Pleass *et al.*, 1996). Fifty microliters of a PCR reaction was run on a 2% agarose gel. The bands of interest were excised from the gel, purified using the QIAquick Gel Extraction Kit (Qiagen Inc.) and ligated into the pCR2.1 vector (Invitrogen Corp., Carlsbad, CA). After transformation into the appropriate *Escherichia coli*, colonies from each sample were expanded. Plasmid DNA was screened on a 1% agarose gel after *EcoRI* digestion, to confirm the presence of the correct fragment size. All DNA sequences were determined using the ABI Prism Dye Termination Cycle Sequencing kit (PerkinElmer, Inc., Wellesley, MA) on an ABI model 3100 automated sequencer (PerkinElmer, Inc.). The forward and reverse M13 primers were used for sequencing. Completed sequences were edited and aligned using the MacVector sequence analysis package (Accelrys Inc., Burlington, MA).

Expression of Macaque CD89 cDNAs in HeLa Cells

Full length rhesus and cynomolgus CD89 cDNAs ligated into the pCR2.1 vector were subjected to digestion with *XhoI* and *EcoRI*. The resulting fragments were ligated into the *XhoI* and *EcoRI* digested expression vector pcDNA3.1 (+) (Invitrogen Corp.) and amplified in *E. coli*. Twenty μ g of expression vector was then added to HeLa cells suspended in 250 μ l of Dulbecco's modified Eagle medium (DMEM) at 14×10^6 cells/ml. After ten min of incubation on ice, cells were electroporated by one or two pulses using the power supply set to 300V, 25mA, and 25W and the Electroporator II (Invitrogen

Corp.) set to 1000 μ F and $\infty\Omega$, according to the manufacturer's recommendations. Cells were then incubated at room temperature for ten minutes and grown in 10ml of DMEM with 10% fetal calf serum in 5% CO₂ at 37°C. For rhesus macaque CD89, stably transfected cells were established by selection with Geneticin (400 μ g/ml) added 72 hours post transfection. After several passages non-transfected and transfected HeLa cells were harvested from cell culture following three PBS washes from wells with 90% confluent growth. 1×10^6 cells were stained at 4°C with either 20 μ l of phycoerythrin-conjugated mouse anti-human CD89 (clone A59) or SimulstestTM Control γ_1/γ_{2a} (both from Becton, Dickinson and Co., San Diego, CA) for 15 min, followed by 3 washes with PBS. A59 binds to the extracellular domain 2 (EC2) of CD89 (Morton *et al.*, 1999). Cells were then fixed with 1% paraformaldehyde and analyzed using a FACSCalibur flow cytometer (Becton, Dickinson and Co.). Repeated flow analysis of cells transfected for rhesus CD89 confirmed that cells stably expressed rhesus CD89. Expression of cynomolgus macaque CD89 in HeLa cells was determined as described above, with the exception that only transient transfectants were generated and examined by flow cytometry analysis.

Determination of CD89 Expression on Blood Leukocytes

Blood from 4 rhesus and 7 cynomolgus macaques was collected in EDTA Vacutainer® tubes (Becton, Dickinson and Co.) by venipuncture under anesthesia. Leukocyte expression of CD89 was analyzed by two-color flow cytometry analysis using phycoerythrin (PE)-conjugated anti-human CD89 and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for CD14, CD20, and CD3 and cy-chrome conjugated

anti-CD16 (Becton, Dickinson and Co.). Monoclonal antibody clones for CD14, CD20, CD3 and CD16 were M5E2, L27, SP34, and 3G8 respectively. SimultestTM Control γ_1/γ_{2a} was used to detect non-specific binding of mouse IgG to cells. Staining of whole blood was done using a standard procedure. Briefly, 100 μ l of blood was incubated with 20 μ l of each antibody in the dark at room temperature. Erythrocytes were lysed with 2ml of BD PharM Lyse (Becton, Dickinson and Co.), washed 3 times with PBS and fixed with 1% paraformaldehyde. 5000 events were counted by flow cytometry.

Results

We cloned and sequenced macaque cDNA obtained through reverse transcription of total RNA followed by amplification performed using primers complementary to sequences located in the S1 and TM/C exons. The introduction of errors into the sequence was minimized by using a high fidelity polymerase with proofreading ability. To validate the amplification strategy, we amplified, cloned and sequenced the full length human CD89 as well as two human alternatively spliced variants. All human sequences matched those available in GenBank (accession numbers NM_002000, NM_133271 and NM_133279) (data not shown). We then used the same strategy to amplify, clone and sequence rhesus and cynomolgus macaque CD89. Each sequence was confirmed by cloning and sequencing the products of another independent PCR performed using the same total RNA. A complete transcript including all five exons along with several additional transcripts representing alternatively spliced forms of the CD89 mRNA were identified. Figure 2.1 shows the deduced amino acid sequences of the complete cDNA

from rhesus and cynomolgus macaques along with the corresponding human sequences. All five macaque exons were of length identical to the corresponding human CD89 exons. The rhesus macaque and the cynomolgus macaque CD89 amino acid sequences exhibit 86.5% and 86.1% identity to the human counterpart, respectively. The rhesus macaque CD89 amino acid sequence shows 99.3% identity to the corresponding cynomolgus macaque sequence. Therefore, the CD89 sequences from these two nonhuman primate species are highly homologous to each other, differing for only two amino acids (a methionine/isoleucine substitution at position 40 and a phenylalanine/leucine substitution at position 85). We did not identify amino acid differences in the S1 and S2 exons between human and macaque sequences. The majority of human/macaque substitutions are clustered in the EC1 exon.

To ascertain whether or not expression of the isolated full-length CD89 cDNA resulted in the production of a cell surface product, we generated HeLa cell transfectants using plasmids containing either rhesus or cynomolgus macaque full-length CD89 cDNA. Transfected cells were stained with a PE conjugated anti-human CD89 and a mouse isotype control and analyzed by flow cytometry. Transfected cells exhibited increased fluorescence intensity when stained for CD89 as compared to staining with a control mouse antibody (Figure 2.2). Staining with anti-human CD89 PE did not result in detectable fluorescence of untransfected HeLa cells indicating that CD89 was not expressed in these cells prior to introduction of the expression vectors. The lower fluorescence intensity levels observed in cynomolgus macaques as compared to rhesus

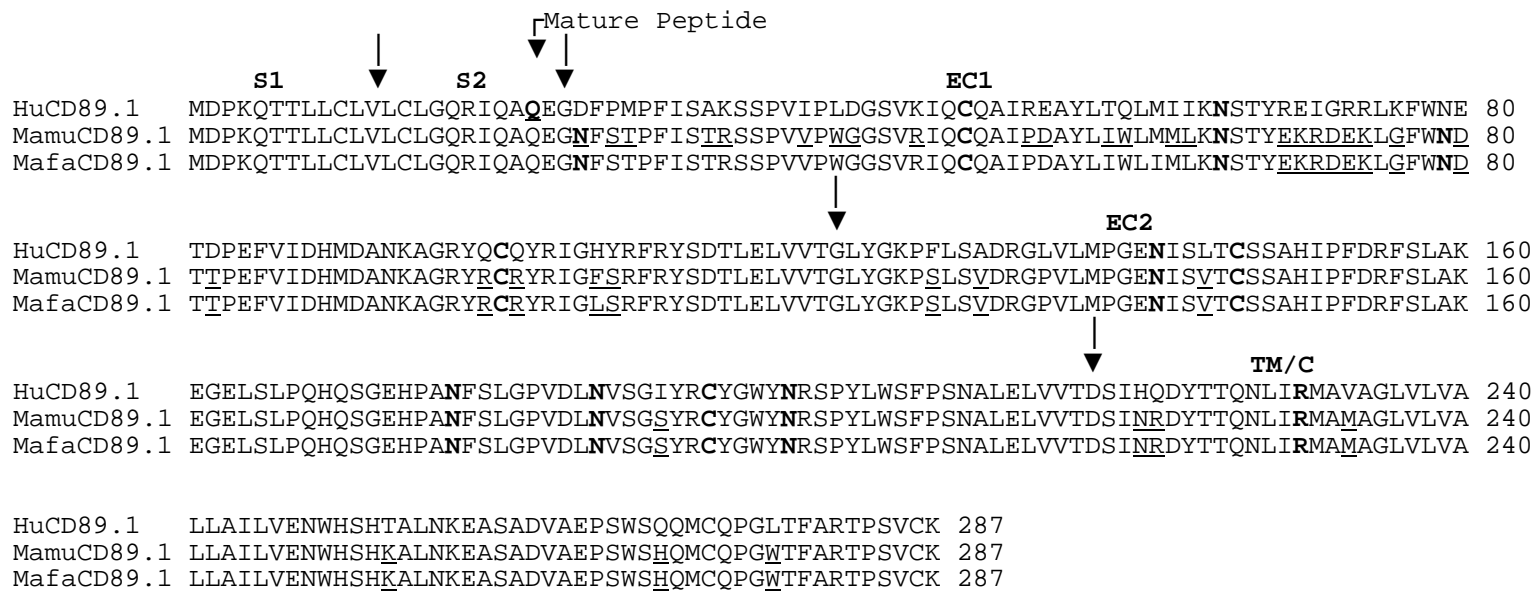


Figure 2.1. Alignment of CD89 derived amino acid sequences obtained by cloning and sequencing rhesus macaque (GenBank accession number AY386684) and cynomolgus macaque (GenBank accession number AY386690) cDNA from whole blood and comparison with the published human sequence (GenBank NM_002000). Amino acid differences are underlined. The first amino acid of the preprotein is numbered as residue 1. The mature peptide starts at residue 22. Arrows indicate distinct domains. The first two amino acids for EC1 are encoded at the end of the S2 exon. The signal peptide is encoded by both S1 and S2 sequences. Potential N-glycosylation sites, cysteines involved in disulfide bonds and arginine 209 critical for association with the FcRγ chain are bolded. Hu: *Homo sapiens*; Mamu: *Macaca mulatta*; Mafa: *Macaca fascicularis*.

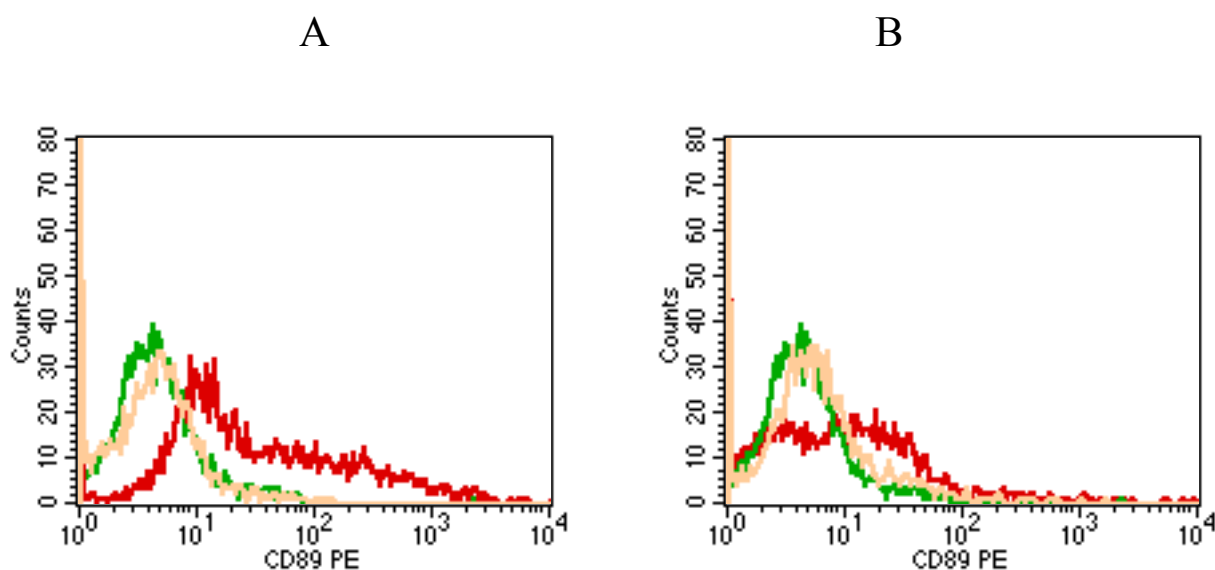


Figure 2.2. Expression of recombinant macaque CD89 on HeLa cells. Cells were transfected with a plasmid containing full-length (A) rhesus macaque CD89 cDNA or (B) cynomolgus macaque CD89 cDNA and stained with anti-human CD89: red line (rhesus MFI = 120.70, SD= 343.04; cynomolgus 30.35, SD=345.04) or with isotype control mouse IgG: tan line (rhesus MFI = 8.65, SD=7.40; cynomolgus MFI=9.27, SD=34.47). Untransfected HeLa cells stained with anti-human CD89: green line (MFI = 5.26, SD= 28.69). MFI= Mean fluorescence intensity, SD= standard deviation. Stable transfectants were used for detection of rhesus macaque CD89 and transient transfectants were used for detection of cynomolgus macaque CD89. 5,000 events were counted per sample.

macaques are likely to reflect the transient transfection of HeLa cells with the cynomolgus macaque cDNA (as mentioned above, stable transfectants were used to detect cell surface expression of rhesus macaque CD89).

Several distinct mRNA isoforms of the human CD89 have been identified. These isoforms are generated through deletion of exons or parts of exons via alternative RNA splicing, resulting in the expression of closely related but functionally different receptor variants (Morton *et al.*, 1996; Patry *et al.*, 1996; Pleass *et al.*, 1996; Reterink *et al.*, 1996). Therefore, alternative splicing may represent the mechanism underlying the diversification of CD89 functions (Pleass *et al.*, 1996). Ten human variants are listed in the GenBank data base. In addition to the complete CD89 transcript, we have identified several alternatively spliced transcripts in macaques. Specifically, five splice variants were present in the rhesus macaque blood sample analyzed and three splice variants in the cynomolgus macaque sample. Figure 2.3 depicts the derived amino acid sequences of the rhesus macaque variants. Three of the five variants (MamuCD89.3, MamuCD89.7 and MamuCD89.9) are similar to described human CD89 isoforms (Monteiro and van de Winkel, 2003), whereas two variants (MamuCD89. Δ EC1 and MamuCD89. Δ EC1 Δ TM/C) have not been described in humans. The derived amino acid sequences of the splice variants from cynomolgus macaques are shown in Figure 2.4. Of the three isoforms, one (MafaCD89.9) is present also in humans and rhesus macaques, whereas the other two (MafaCD89. Δ EC1 and MafaCD89. Δ EC1 Δ TM/C) are shared with rhesus macaques but not humans. Figure 2.5 shows the schematic representation of the complete CD89 transcript and corresponding splice variants identified in both macaque species.

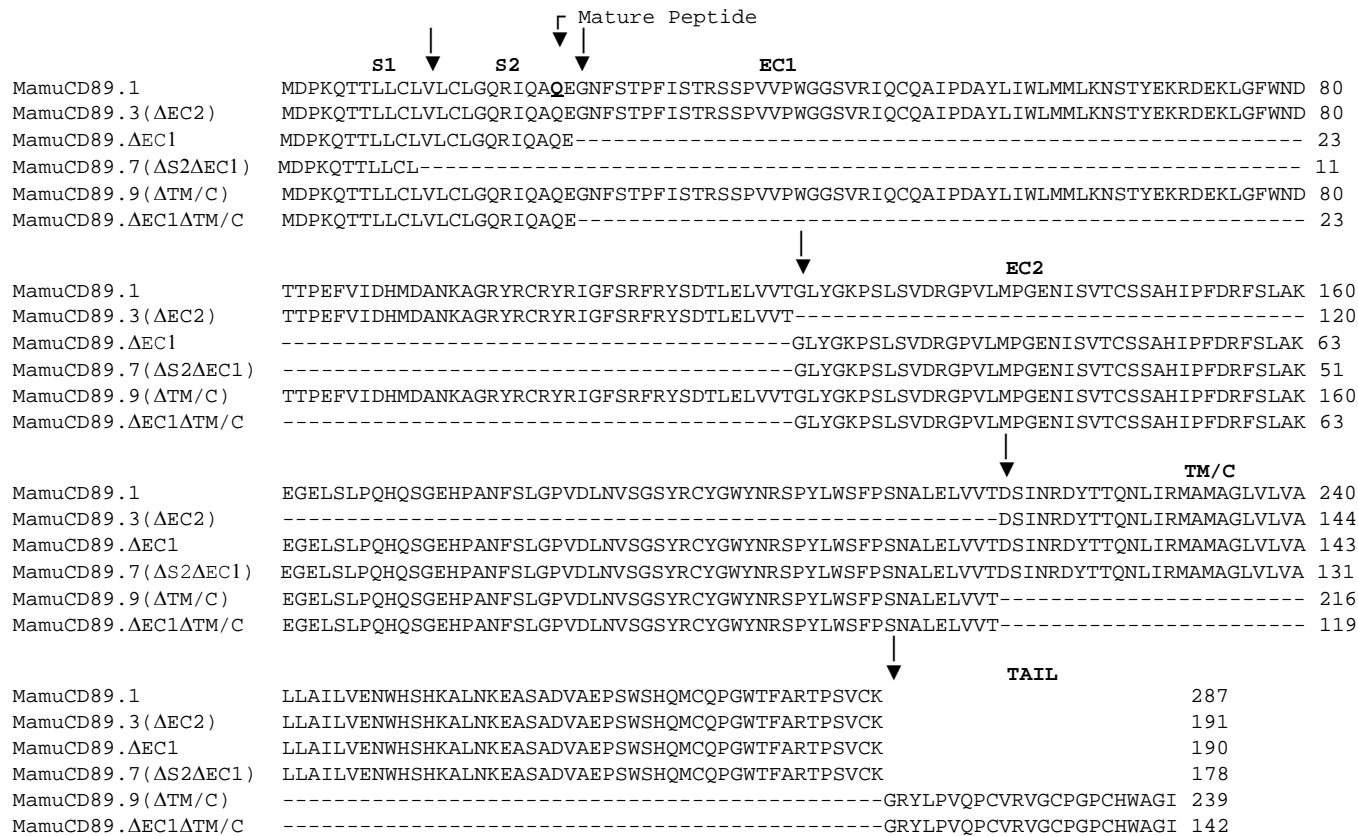


Figure 2.3. Alignment of the five rhesus macaque CD89 splice variants (GenBank accession number AY386684-AY386689) with full-length CD89 from the same species. Arrows indicate distinct domains. Mamu: *Macaca mulatta*.

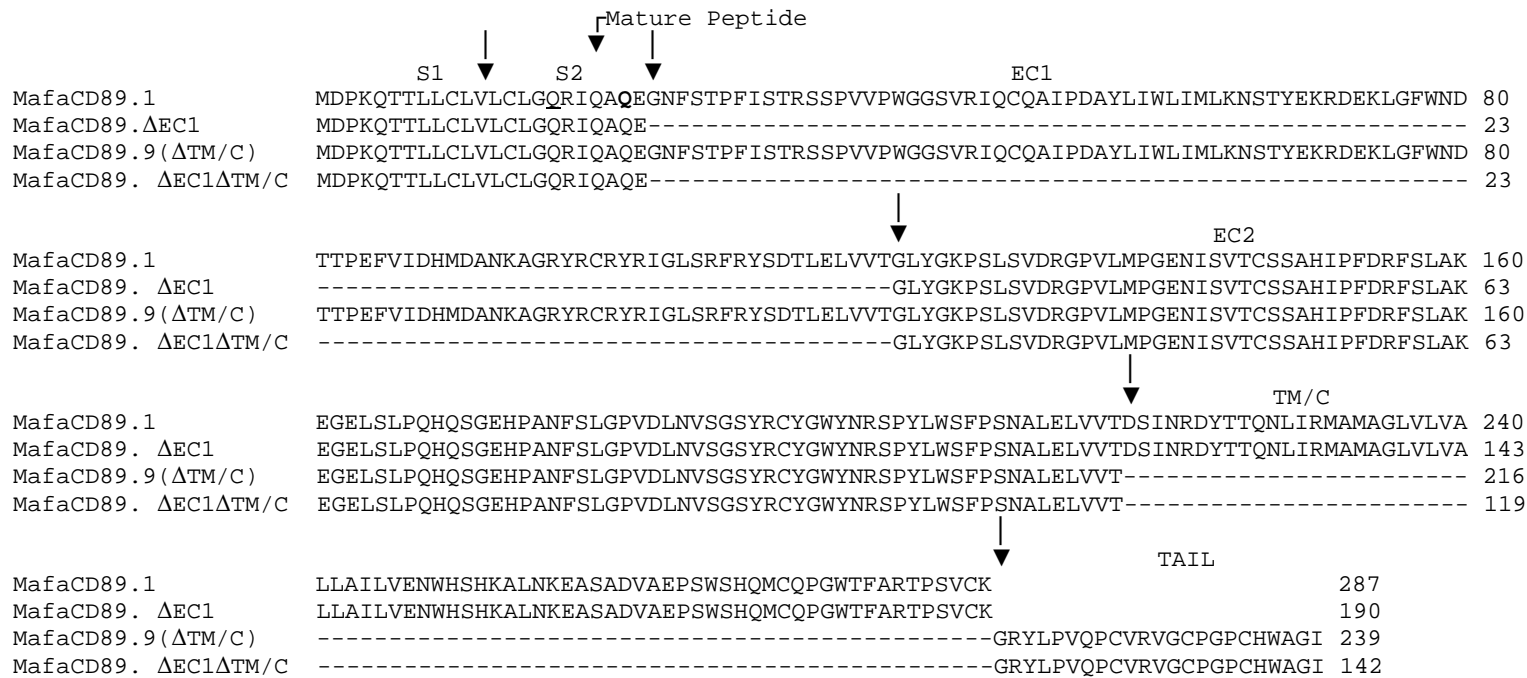


Figure 2.4. Alignment of the three cynomolgus macaque CD89 splice variants (GenBank accession number AY386690-AY386693) with full-length CD89 from the same species. Arrows indicate distinct domains. Mafa: *Macaca fascicularis*.

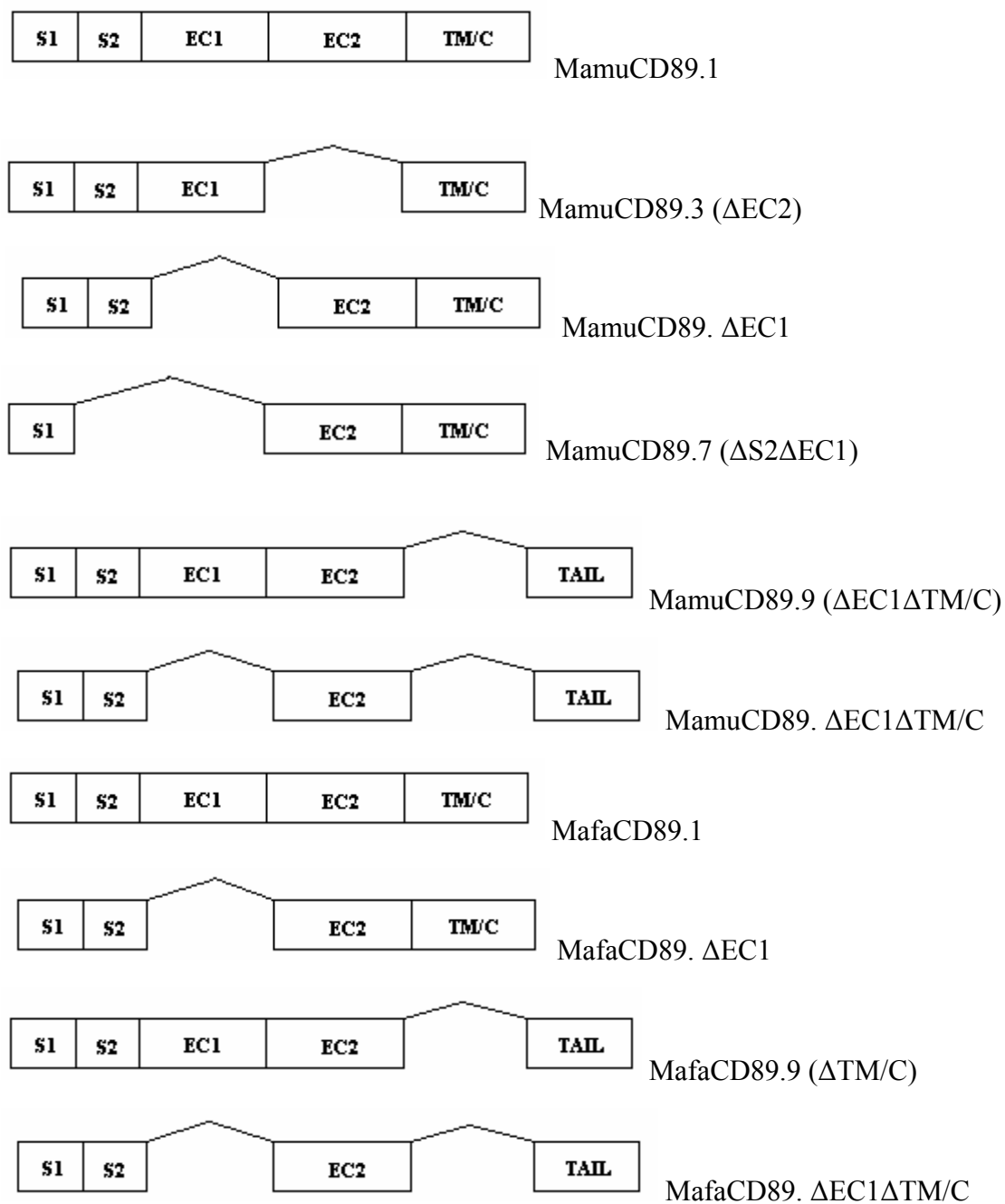


Figure 2.5. Schematic representation of the complete CD89 transcript and corresponding splice variants identified in rhesus and cynomolgus macaques. Mamu: *Macaca mulatta*; Mafa: *Macaca fascicularis*.

The two variants identified in macaques and not described in humans are characterized by a deletion of the EC1 exon or by a deletion of the EC1 and TM/C exons. The latter variant maintains the tail sequences, as found in a human isoform with deletion of the TM/C exon. This human isoform is designated Fc α RIb and results from an alternate splicing that, by skipping the 3' splice site located at the end of the exon EC2, introduces a tail of 23 new amino acids before reaching the stop codon (van Dijk *et al.*, 1996).

Human CD89 is expressed only in selected cells of the myeloid lineage and not in lymphocytes. To determine whether or not macaque CD89 is similarly expressed, whole blood from four rhesus and seven cynomolgus macaques was stained with the anti-human CD89 PE as well as various FITC-conjugated antibodies against markers of leukocyte populations and then analyzed by flow cytometry. Both macaque species expressed CD89 on granulocytes and monocytes, but not on lymphocyte populations. Cells positive for markers of B cells, T cells and natural killer cells (CD20, CD3 and CD16 respectively) were all CD89 negative in both species (Figure 2.6). In humans, CD16 is present on neutrophils as well as on natural killer cells (Fleit *et al.*, 1982). However, our results indicate that either macaque granulocytes do not express CD16 or granulocyte CD16 is present in a form not recognized by the antibody clone used in our study. Rhesus macaque leukocytes stained for CD14 can be plotted along with their side scatter properties to distinguish the lymphocyte, eosinophil, neutrophil, and monocyte populations (Lafont *et al.*, 2000). Taking advantage of this, we stained whole blood for CD89 and CD14, and gated cell populations using a sidescatter versus CD14 dot plot (Figure 2.7). Without gating, CD14/CD89 staining identified three clusters of cells: a

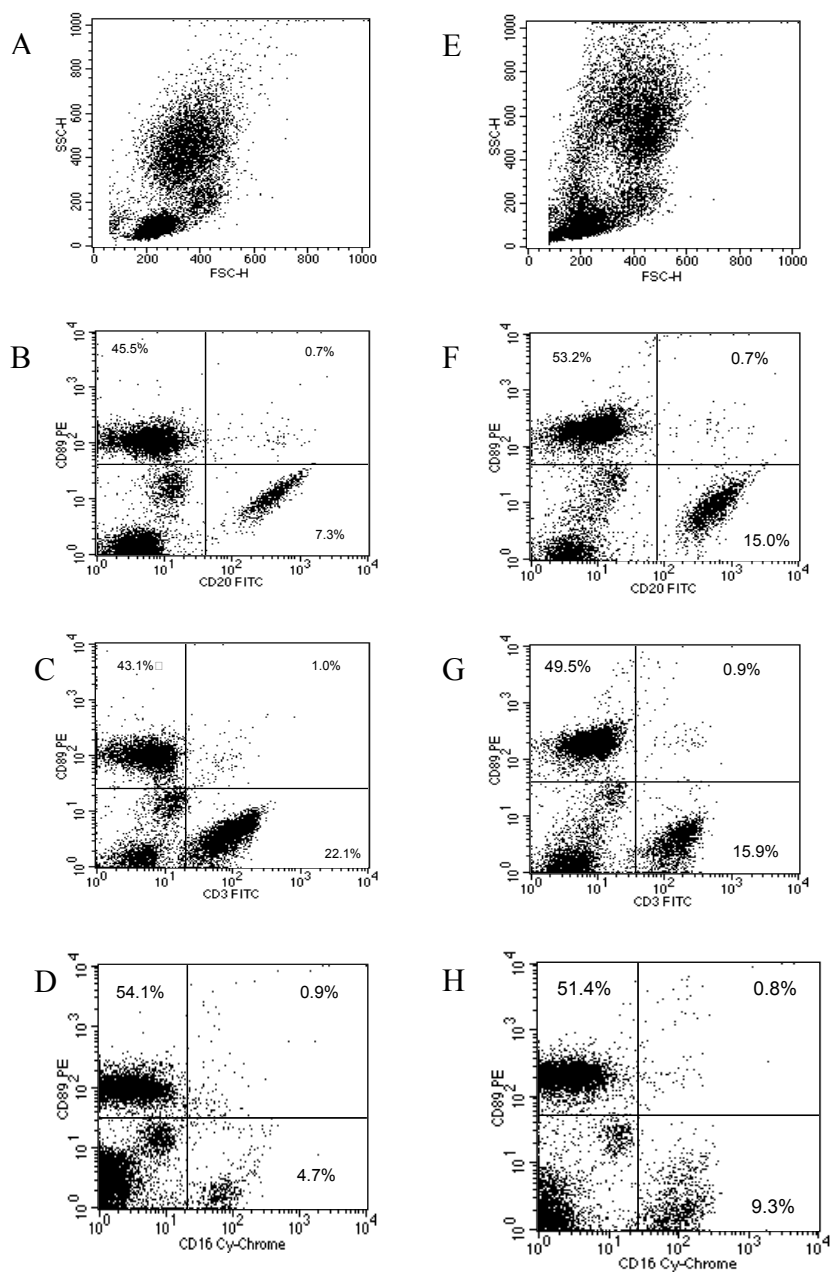


Figure 2.6. CD89 is not expressed on lymphocytes of rhesus and cynomolgus macaques.

Two-color dot-plots of whole blood leukocytes from a representative rhesus (A-D) and cynomolgus macaque (E-H). (A and E) forward scatter (FSC) versus side scatter (SSC). CD89 versus CD20 (B and F), CD3 (C and G), and CD16 (D and H).

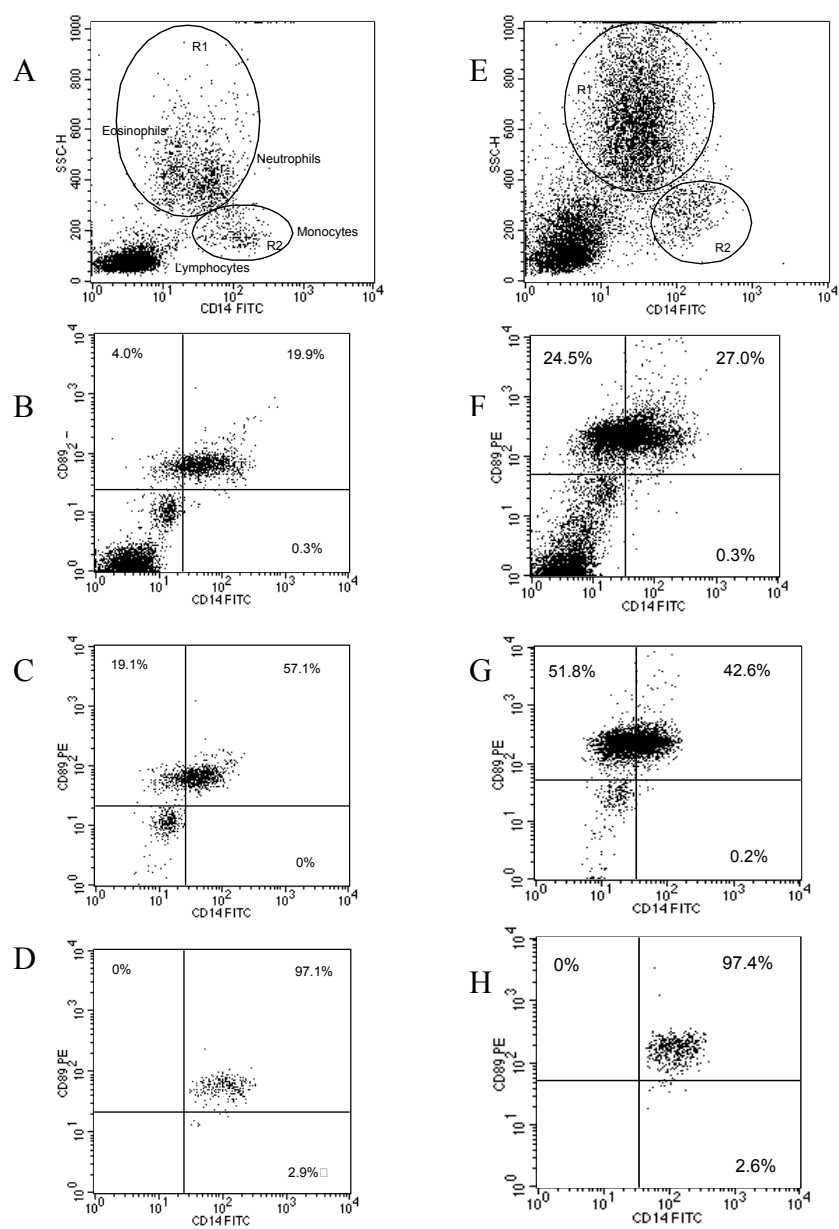


Figure 2.7. CD89 is expressed on monocytes and on granulocytes of rhesus and cynomolgus macaques. Two-color dot-plots of whole-blood leukocytes from a representative rhesus (A-D) and cynomolgus macaque (E-H). (A and E) CD14 versus side scatter. CD14 versus CD89 (B and F) total leukocytes, (C and G) granulocytes, (D and H) monocytes. Gates used for granulocyte (R1) and monocyte (R2) populations of rhesus (A) and cynomolgus macaque (E) are shown.

double negative population in the bottom left hand corner corresponding to lymphocytes, a population with intermediate fluorescence on both axes, and a cluster of cells positive for CD89 with a broad range of expression for CD14. Gating of the granulocyte population revealed that the latter two populations contained granulocytes. The cluster of cells with intermediate fluorescence likely corresponds to a portion of eosinophils, as eosinophils are known to exhibit greater autofluorescence than other cell populations (Lafont *et al.*, 2000). A percentage of the eosinophil population was always found in the CD89 high fluorescence cluster of cells, with the remaining CD89 positive cells representing neutrophils and monocytes.

Discussion

Macaques are widely used in biomedical research as models for pathogenesis studies, vaccine development and testing of immunotherapeutic approaches, including experimental strategies to prevent transplant rejection (Attanasio *et al.*, 2000; Guirakhoo *et al.*, 2000; Solnick *et al.*, 2000; Hahn *et al.*, 2001; Asiedu *et al.*, 2003; Custer *et al.*, 2003; Lu *et al.*, 2003; Vugmeyster *et al.*, 2003; Xu *et al.*, 2003). It is well recognized that macaques infected with simian immunodeficiency viruses (SIV) or simian-human immunodeficiency viruses (SHIV) are the best animal model currently available to study AIDS pathogenesis and vaccine development (Hirsch and Lifson, 2000). Given the importance of CD89 in the immune response, we identified and characterized CD89 cDNA in two different macaque species. Results from our experiments show that HeLa cells transfected with plasmids containing rhesus macaque or cynomolgus macaque

CD89 cDNA express the CD89 molecule on their cell surface, and this molecule is recognized by an anti-human CD89 antibody. In addition, our results indicate that, similarly to the human counterpart, macaque CD89 is expressed on blood leukocytes of the myeloid lineage.

The rhesus and cynomolgus macaque CD89 amino acid sequences exhibit 86.5% and 86.1% identity with the human counterpart, respectively, and are highly homologous to each other (99.3% identity). The human CD89 cDNA encodes a protein containing six potential N-glycosylation sites, four of which are located in the extracellular domains (Maliszewski *et al.*, 1990). The presence of ordered carbohydrates at these four sites (N44, N58, N120, and N156) has been recently demonstrated in the crystal structure of the human CD89 (Herr *et al.*, 2003). The other two sites are located at positions 165 and 177. Differentially glycosylated CD89 species are expressed on monocyte/macrophages and granulocytes (Monteiro *et al.*, 1992). As shown in Figure 2.1, the six glycosylation sites are also present in the CD89 sequences from both macaque species. However, an additional potential glycosylation site is present in the EC1 domain of the macaque CD89 (asparagine at position 4). In the human CD89 molecule, cysteines involved in disulfide bonds are located at positions 28 and 79 of the EC1 domain and at position 125 and 172 of the EC2 domain. As expected, these four cysteines are conserved in the CD89 sequences from both macaque species. Additionally, arginine 209, critical for CD89 association with the signaling molecule FcR γ chain (Morton *et al.*, 1995), is conserved in macaques.

Human CD89 interacts with human IgA molecules through residues located on the EC1 domain. These residues, which include Y35, Y81 and R82 (along with R52 and to a lesser extent H85 and Y86), appear to form a single face at the N-terminus of the molecule and have been identified by scanning mutagenesis (Wines *et al.*, 1999; Wines *et al.*, 2001). While Y35 and Y81 are conserved in both macaque species, the arginine at position 52 and the tyrosine at position 86 are substituted by a glutamic acid and a serine, respectively, in both macaque species. In addition, the histidine located at position 85 of the human sequence is replaced by a phenylalanine in rhesus macaques and by a leucine in cynomolgus macaques. Recently, additional human CD89 residues involved in IgA binding (along with those previously described) have been identified by analyzing the crystal structure of the molecule (Herr *et al.*, 2003). Of these amino acids, L54, F56, W57 and G84 are conserved in the human and macaque CD89 molecules, whereas the arginine at position 53 and the lysine at position 55 of the human molecule are substituted by a lysine and by a glycine, respectively, in both macaque species. To date, the only CD89 homologue model for IgA binding is the recently described bovine CD89 (bCD89), which was found to bind human and bovine IgA similarly (Morton *et al.*, 2004). Binding of macaque IgA to macaque CD89 has not yet been shown. Interestingly, two of the human CD89 residues identified in the crystal structure as contributing to IgA binding (R53 and K55) are lysine and glycine, respectively, in both bovine and macaque CD89. This and the observation that bCD89 is more dissimilar than macaque CD89 to human CD89 indicates that, although some of the amino acids involved in the human IgA/CD89

interaction are not conserved in macaques, macaque CD89 is likely to bind IgA from the same species.

Lack of identification in the macaque samples of additional splice variants similar to human transcripts may be due to different factors. Macaques may express only a limited number of the isoforms described in humans or, most likely, the identified macaque isoforms are a reflection of the samples used for RNA purification and therefore of transcripts that are present in cells found in peripheral blood. Human CD89 splice variants are indeed cell-type specific. The CD89.2 isoform is found only in alveolar macrophages (Patry *et al.*, 1996). Similarly, expression of the CD89.3 variant (Δ EC2) is cell-type specific (Togo *et al.*, 2003). The presence of variants found only in macaques may indicate that such variants are specific to these species or that the corresponding human isoforms have not been identified yet. In addition, it has been demonstrated that the ratio of variants to wild type CD89 is regulated by inflammatory cytokines and can be differentially modulated by diseases (Monteiro *et al.*, 2002; Togo *et al.*, 2003). Indeed, we have observed differences in the relative intensity of bands for cDNA of the various splice variants from an individual varied compared with other individuals (data not shown). Therefore, CD89 isoform expression may be dependent on the particular individual analyzed and on the concomitant presence of inflammatory conditions or other disorders. Clearly, additional studies are necessary to fully characterize the entire set of macaque CD89 splice variants.

The IgA Fc receptor has been characterized only in a few species. The rat CD89 gene homologue shares 53% amino acid identity with the human CD89 (Maruoka *et al.*,

2004). Only full-length CD89 and a single splice variant (characterized by a deletion of the S2 exon) have been identified in rat spleen (Maruoka *et al.*, 2004). The cattle CD89 homologue shares 56.2% amino acid identity with the human CD89. However, no splice variants have been reported in this species (Morton *et al.*, 2004). The identification of several variants in macaques indicates that CD89 transcript processing may vary considerably between species.

There is currently increased interest in the manipulation of the IgA/CD89 interaction for immunotherapeutic purposes (Dechant and Valerius, 2001; Corthesy, 2002; Presta, 2002). CD89 represents an effective target molecule for immunotherapy mediated by bispecific antibodies (Valerius *et al.*, 1997). Results from a more recent study show that a chimeric surfactant protein D/anti-CD89 protein may effectively target pathogens to neutrophils (Tacke *et al.*, 2004). Like humans, macaques express CD89 on granulocytes and monocytes. Because of the potential use of macaques as models for the development of IgA-based therapies, the characterization of CD89 and corresponding isoforms in these species represents an essential step to define a valuable system for the testing of therapeutic antibodies.

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CHAPTER 3

Immunoglobulin A Fc receptor I (CD89) Homologues in Nonhuman Primates: Identification and Characterization of Baboon and Sooty Mangabey CD89 and Characterization of Rhesus Macaque CD89 Interactions with Human and Rhesus Macaque IgA

Keywords: IgA, CD89, IgA receptor, Nonhuman primate models

Summary

Immune complexes containing IgA interact with the IgA Fc receptor I (CD89) to initiate the pathogen elimination processes carried out by cells of the myeloid lineage. Results from *in vitro* studies using IgA bispecific antibodies directed to either tumor or pathogen antigens and to CD89 show the great potential of therapeutic antibodies capable of harnessing immune responses. In addition, IgA/CD89 immune complexes appear to contribute to IgA nephropathy, the leading cause of first stage renal failure. Therefore, the characterization of IgA/CD89 interactions is essential to understand both physiological and pathological mechanisms mediated by these interactions. Currently, there is no established *in vivo* model for studying IgA/CD89 interactions. We have previously characterized the CD89 blood leukocyte expression profile in macaques and identified macaque CD89 genes. Here, we have extended our studies by characterizing CD89 gene transcripts and protein expression patterns in two additional nonhuman primate species important in biomedical research, baboons and sooty mangabeys.

Furthermore, we have employed recombinant rhesus macaque IgA and recombinant CD89 molecules to evaluate macaque IgA/CD89 binding properties. Our results show that leukocytes from baboons and sooty mangabeys produce several alternatively spliced CD89 gene transcripts. In these species, CD89 is detected only on monocytes and on granulocytes. Multiple CD89 alleles that fit a Mendelian mode of inheritance are present in baboons. Macaque CD89 binds to macaque IgA as well as to human IgA1 and IgA2. This binding is nearly abolished by blocking N-glycosylation. We have expressed two macaque CD89 isoforms produced from alternatively spliced transcripts in HeLa cells. One of these isoforms appears to be secreted and is capable of interacting with IgA complexes. This interaction reduces initial binding of IgA to CD89 expressed on HeLa cells and may act *in vivo* to block cross-linking of CD89 by IgA, thus resulting in inhibition of cellular activation. Together, these results greatly contribute to the development of animal models for studying the function of serum IgA, the mechanisms involved in the pathogenesis of IgA nephropathy, and the therapeutic potential of antibodies that interact with CD89 molecules.

Introduction

The human immunoglobulin A (IgA) Fc receptor I (CD89) is expressed on neutrophils, monocytes, and other select myeloid lineage cells. Cross-linking of CD89 with IgA-opsonized pathogens initiates a variety of cellular immune responses, including phagocytosis, that assists in killing and removing these pathogens. In the absence of antigen binding IgA can act through CD89 to suppress activation of coexpressed IgG Fc receptors, thus helping to explain the anti-inflammatory properties of this

immunoglobulin isotype (Pasquier *et al.*, 2005). CD89 is not present in mice (Maruoka *et al.*, 2004). CD89 homologues have been identified in few species (cow, horse, rat, macaques and chimpanzee) (Maruoka *et al.*, 2004; Rogers *et al.*, 2004; Morton, 2005; Morton *et al.*, 2005). However, these homologues are still poorly characterized. Even human CD89 is incompletely characterized.

As discussed in chapter 2, we have recently identified rhesus and cynomolgus macaque CD89 (Rogers *et al.*, 2004). The whole blood leukocyte expression profile of these receptors matches that of humans. Macaques produce several alternatively spliced transcripts of CD89, including unique transcripts and transcripts similar to those generated by human cells. Macaque and human CD89 are highly conserved. However, they differ for few amino acid substitutions at positions that in humans are necessary for interactions with IgA. To further our understanding of CD89 structure and function, we have identified and characterized CD89 full length transcripts and several splice variants from baboons and sooty mangabeys. In addition, we show that recombinant rhesus macaque CD89 generated in HeLa cells is able to specifically bind human IgA1 and IgA2 as well as recombinant rhesus macaque IgA. Efficient expression of rhesus macaque CD89 in HeLa cells is dependent on N-glycosylation.

Materials and methods

Animals

Blood samples were collected from a total of five healthy sooty mangabeys (*Cercocebus torquatus*) and twenty healthy baboons (*Papio hamadryas anubis*). The sooty mangabey samples were from animals housed at the Yerkes National Primate Research Center, Emory University, Atlanta, GA. Baboon samples were from animals housed at the Southwest National Primate Research Center San Antonio, TX. The animals used for sequencing of the CD89 gene were different from those used for flow cytometric analysis of blood leukocyte expression of CD89.

Amplification, cloning and sequencing of baboon and sooty mangabey CD89

Heparinized blood samples were collected from one sooty mangabey (*Cercocebus torquatus*) and sixteen baboons (*Papio hamadryas anubis*). Total RNA was extracted from whole blood using the QIAamp RNA Blood Mini Kit (Qiagen Inc., Valencia, CA), and reverse transcribed into cDNA using oligo d(T)17 primers, followed by primer extension with the AMV reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN). Reverse transcription PCR amplification, cloning and sequencing were performed following a previously described protocol to identify CD89 in primates (Rogers *et al.*, 2004). PCR forward (RP1) and reverse (RP2) primers used to amplify the full-length CD89 transcripts have been previously described (Pleass *et al.*, 1996). Forward (RP1) and reverse (RP2) primers specific for human CD89 are located at the start codon of the S1 exon and the stop codon of the TM/C exon, respectively. An

alternate form of CD89, known as Fc α RIb or CD89.9 (which lacks exon TM/C but contains nucleotide sequences present in the intron located between exon EC2 and exon TM/C) has been described for humans and macaques (van Dijk *et al.*, 1996; Rogers *et al.*, 2004). Baboon and sooty mangabey splice variants of this type were amplified here as described previously with the reverse primer FCARB2 and forward primer RP1, which yield full-length Fc α RIb transcripts (Rogers *et al.*, 2004). Each sequence was confirmed by cloning and sequencing the products of another independent PCR performed using the same total RNA.

Analysis of DNA sequences

Overlapping regions were identified and sequences were edited using the MacVector software program (Accelrys Inc., San Diego, CA). Sequences were aligned with each other and other known CD89 genes using the CLUSTAL function of the MEGALIGN part of the LASERGENE software package (DNASTAR Inc., Madison, WI). The GenBank accession numbers for the CD89 sequences previously published are human NM_0020000, chimpanzee BK005386, rhesus macaque AY386684, cynomolgus macaque AY386690, rat AB109767, cow AY247821, pig CR45038 and horse AY587560. The pig gene has not previously been identified. We have predicted its sequence from a pig contig sequence on the basis of sequence homology with other CD89 genes.

Determination of CD89 expression on blood leukocytes

Blood from 4 baboon and 4 sooty mangabeys was collected in EDTA Vacutainer® tubes (Becton, Dickinson and Co., San Jose, CA) by venipuncture under anesthesia. Mangabey leukocyte expression of CD89 was analyzed by two-color flow cytometry using phycoerythrin (PE)-conjugated anti-human CD89 (clone A59) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD16 (clone 3G8) (Becton, Dickinson and Co.). Baboon leukocyte expression of CD89 was analyzed by staining with PE anti-human CD16 alone. Simultest™ Control γ_1/γ_1 was used to detect non-specific binding of mouse IgG to cells (Becton, Dickinson and Co.). Staining of whole blood was done using a standard procedure. Briefly, 100 μ l of blood was incubated with 20 μ l of each antibody in the dark at room temperature for 15 min. Erythrocytes were lysed with 2ml of BD PharM Lyse (Becton, Dickinson and Co.), washed 3 times with PBS and fixed with 1% paraformaldehyde. 10000 cells were counted by flow cytometry. Samples were read using a FACSCalibur flow cytometer and data analyzed using BD CellQuest™ Pro version 5.2 software (Becton, Dickinson and Co.).

Generation of HeLa cell clones expressing recombinant rhesus macaque CD89

Previously we generated recombinant rhesus macaque CD89 in HeLa cells using a vector constructed with pcDNA3.1 (Invitrogen Corp., Carlsbad, CA) (Rogers *et al.*, 2004). However, even after extensive selection with G418, expression of CD89 using this vector in HeLa cells was only temporary. To improve the stability of the expression vectors in transfected cells, part of the pcDNA3.1 constructs (containing cDNA of rhesus

macaque CD89, a cytomegalovirus promoter, a bovine growth hormone polyadenylation signal, and a fl origin) were amplified and inserted into the vector pLSXN (Clontech Laboratories, Inc., Mountain View, CA), which contains long terminal repeats for integration into chromosomes. pLSXN contains a neomycin resistance gene, which allows for transfectant selection. Insertion of a pcDNA-CD89 fragment into pLSXN was carried out by amplifying with primers PcHp (5'-CTGCTGTTAACCGTTAGGGTTAGGCGTTTTGCG-3) and PcSa (5'-ACTTTGTCGACGCTCAGCGGCCGGCCATCGATCCACAGAATTAATTCGCGTT-3). The resulting fragment was then digested with *Hpa I* and *Sal I*, whereas pLSXN was digested with *Hpa I* and *Xho I*. The two fragments were then ligated together to form pLSXN-CD89. The *Sal I* digested fragment can be ligated to the *Xho I* digested vector because both enzymes produce the same sticky ends. Similarly, vectors were generated for rhesus macaque CD89 splice variants CD89.9 Δ TM/C and CD89 Δ EC1 from expression vectors created with pcDNA 3.1 according to the method used to generate the original CD89 expression vector with pcDNA3.1 (Rogers *et al.*, 2004).

Large quantities of vector for transfection were prepared using EndoFree® Plasmid Maxi kits (Qiagen, Inc.). Twenty μ g of expression vector was added to HeLa cells suspended in 250 μ l of Dulbecco's modified Eagle medium (DMEM) at 14×10^6 cells/ml. After 10 min of incubation on ice, cells were electroporated by one pulse using the power supply set to 300V, 25mA, and 25W and the Electroporator II (Invitrogen Corp.) set to 1000 μ F and $\infty\Omega$, according to the manufacturer's recommendations. Cells were then incubated at room temperature for 10 min and grown in 10ml DMEM

(containing 10% FCS) in 5% CO₂ at 37°C. Antibiotic G418 (400µg/ml) was added to cells 72 hours post transfection to obtain stable transfectants.

Selection and expansion was performed to isolate HeLa cell clones stably expressing CD89 at high levels. To isolate single clones, cells were diluted serially into 96 well microtiter plates and grown in 100µl of DMEM (containing 10% FCS and consisting of 50% fresh media and 50% conditioned media collected from flasks of untransfected HeLa cells and filtered with a 0.2µm filter) with 400µg/ml G418. Plates were examined by microscopy to identify wells with a single cell. Clones were subsequently expanded in wells of increasing size until cell numbers were sufficient to screen for CD89 expression. Reverse transcription PCR was performed to identify clones with CD89 transcripts. CD89 expression was then verified by staining cells with anti-human CD89 PE (clone A59) or a mouse isotype control, followed by flow cytometric analysis. HeLa cells were prepared for flow cytometry as follows. Adherent cells were removed from flasks with 0.25% trypsin/EDTA and counted. Viability was determined by trypan blue exclusion. Cells were then aliquoted (0.5×10^6 cells/tube), washed with PBS, stained with 5µl of the appropriate antibody for 30 min at 4° C, washed three times with PBS to remove unbound antibody, and resuspended in 1% paraformaldehyde.

Generation of recombinant rhesus macaque IgA

The generation of recombinant rhesus macaque IgA has been previously described (Jayashankar, 2004) (Figure 3.1). This IgA molecule consists of a mouse variable region with specificity for the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) and a Chinese rhesus macaque IgA heavy chain constant region. Expression of recombinant rhesus macaque IgA was performed by transfection of J558L mouse myeloma cells (a gift from Dr. S. L. Morrison, UCLA) with a vector encoding the cDNA from the Chinese rhesus macaque *IGHA* gene (GenBank accession number AY29614; Scinicariello *et al.*, 2004). The vector also contains the mouse immunoglobulin variable heavy region with affinity for the hapten NIP. The complete set of antibody genes includes the variable region of the mouse Ig λ light chain produced in J558L cells (Jayashankar, 2004). J558L cells do not produce a mouse immunoglobulin as a result of a spontaneous loss of the immunoglobulin heavy chain from the parental cells line J558 (Oi *et al.*, 1983). Vector construction was performed in our laboratory by Lakshmi Jayashankar from the parental human genomic IgG3 expression vector pLNOH2 (provided by Drs. Lars Nordehaug and Inger Sandlie, University of Oslo and Antibody Design AS, Nesoddtangen, Norway). pLNOH2 was derived from pcDNA3 (Invitrogen Corp.). To create the final expression vector the human *IGHG3* gene was removed from pLNOH2 by digestion with *HindIII* and *BamHI* followed by ligation with a rhesus macaque *IGHA* cDNA cassette, previously digested with *HindIII* and *BamHI*.

Production of recombinant rhesus macaque IgA from J558L cells was verified by ELISA as follows. Microtiter plates were coated with antigen consisting of

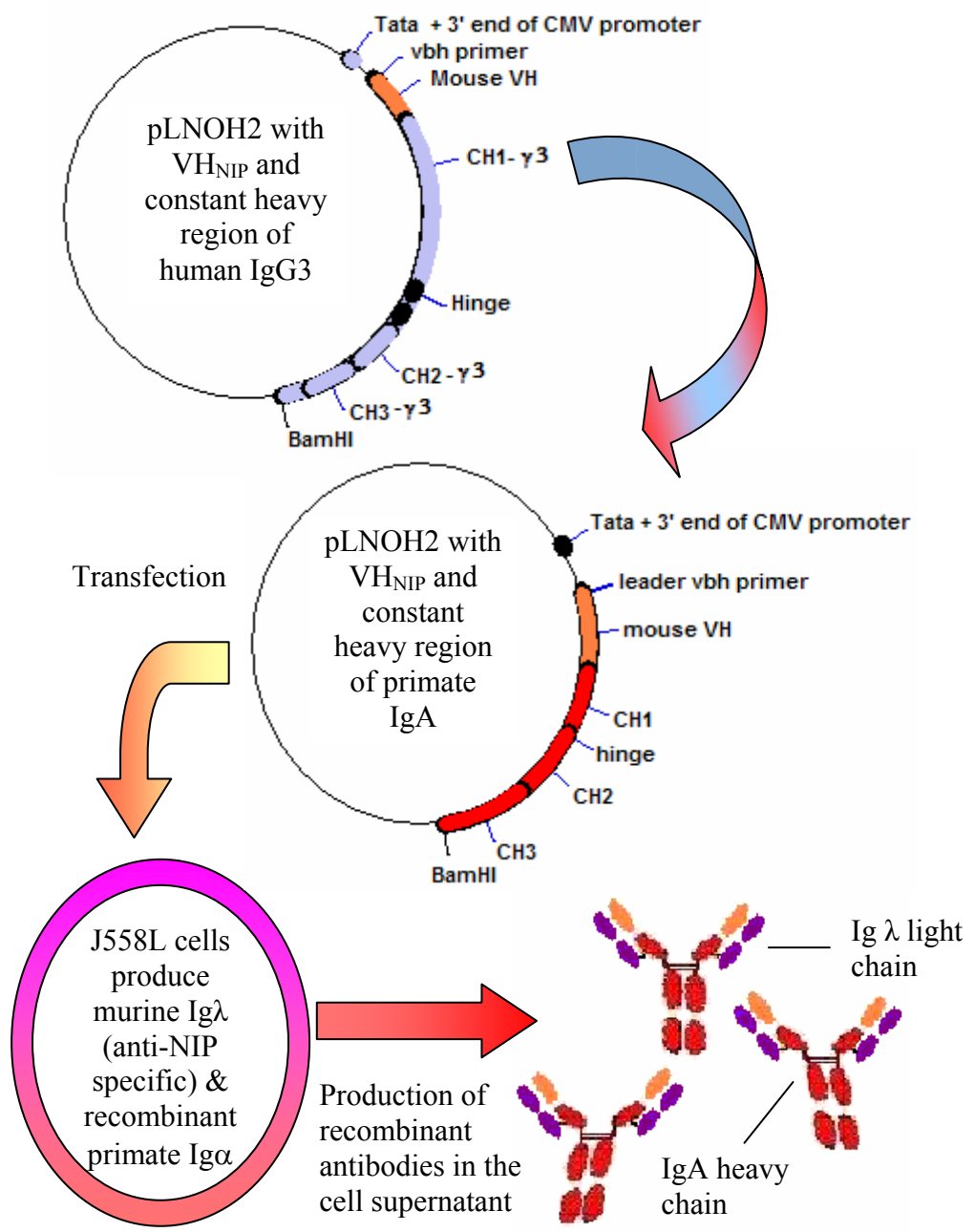


Figure 3.1. General outline of vector construction and expression of nonhuman primate recombinant antibodies. Antibody domains are rhesus: red and mouse: purple and orange.

bovine serum albumin conjugated to five molecules of NIP (NIP (5) BSA) (Biosearch Technologies, Inc., Novato, CA) in coating buffer at 15µg/ml overnight at 4°C. The plates were blocked with 5% FCS at 37°C for 30 min and washed 3 times in PBS with 0.05% Tween 20. One hundred µl of supernatants from the transfected and untransfected cells were added and incubated overnight at 4°C. After washing, 50 µl of 1:2000 HRP-labeled goat anti-rhesus IgA antibody (KPL, Inc., Gaithersburg, MD) and goat anti-mouse Igλ (Invitrogen Corp.) was added. After incubation for 1 hour at 37°C, plates were washed and ABTS/H₂O₂ was added. After addition of the stop solution, absorbance was measured at 405 nm with a Benchmark microplate plate reader (Bio-Rad Laboratories, Inc., Hercules, CA). Samples with the greatest OD values for rhesus macaque IgA were used to examine binding to rhesus macaque CD89.

Immunoglobulin binding assay

Binding of immunoglobulin molecules to CD89 was assessed by flow cytometry using antibodies that were heat aggregated (HA) at 63°C for 1hr. Human myeloma proteins IgA1, IgA2, IgG and IgM (Binding Site Ltd., Birmingham, UK) with immunoglobulin κ light chains were added to 0.5×10^6 cells at 20µg/ml in PBS and incubated for 1 hr at 4°C. For experiments using media from cells transfected with rhesus macaque CD89.9 (CD89 ΔTM/C) and media from untransfected cells, human HA IgA2 or IgM was incubated with cells suspended in media instead of PBS. PBS washed cells were then stained with either FITC-conjugated mouse anti-human Igκ (Invitrogen Corp.), FITC-conjugated mouse anti-human Igα (KPL, Inc.) or FITC-conjugated mouse isotype

control (Becton, Dickinson and Co.) for 30 min at 4°C. For some experiments, noncompetitive mouse anti-human CD89 PE (clone A59) was added. Cells were washed and analyzed by flow cytometry as described above. For N-glycosylation blocking experiments, tunicamycin was added to half of the cell cultures at 1 µg/ml 30 hr prior to harvesting cells. Tunicamycin blocks the addition of N-glycans to proteins by inhibiting the transfer of N-acetylglucosamine-1-phosphate to dolicholmonophosphate.

Experiments to determine binding of recombinant rhesus macaque IgA were performed as variations on the above protocol. Two ml of supernatant from J558L cells secreting recombinant IgA or supernatant from control J558L cells was mixed with NIP (5) BSA (15 µg/ml final concentration) and incubated for 2 hours at 37°C. Cells expressing rhesus macaque CD89 and control HeLa cells were prepared as described above followed by suspension in 50 µl of either supernatant and incubated for 40 min at 4°C. This step was repeated twice more with 20 min incubations following spinning of cells and removal of supernatant each time. Two hundred µl of supernatants without NIP (5) BSA was then added to cells and incubated for 30 min. Finally, cells were washed with PBS, stained with 20 µl goat anti-mouse Igλ FITC and 5 µl anti-human CD89 for 20 min at 4°C, washed 3 times with PBS, and fixed with 1% paraformaldehyde.

Experiments to determine whether or not NIP (5) BSA was necessary for rhesus macaque IgA/CD89 binding were then performed. Three hundred µl of supernatants were added to cells with NIP (5) BSA at concentrations of 15, 7.5, 3.75, 1.88 or 0 µg/ml and incubated at 4°C for 1 hour, then washed, stained and prepared as done above.

Results

Cloning and sequencing of baboon and sooty mangabey full-length CD89 and CD89 splice variants

We cloned and sequenced baboon and sooty mangabey cDNA obtained through reverse transcription of total RNA followed by amplification performed using primers complementary to sequences located in the S1 and TM/C exons. Full-length transcripts including all five exons were identified along with several additional transcripts representing alternatively spliced forms of the CD89 mRNA. Figure 3.2 shows the deduced amino acid sequences of the full-length cDNA from baboons (represented by the sequence most frequently observed in baboons) and sooty mangabeys (one of two variant sequences) along with the corresponding sequences from humans and other primates. All five baboon and sooty mangabey exons were of length identical to the corresponding human CD89 exons. The baboon and the sooty mangabey CD89 amino acid sequences exhibit 86.4% and 87.1% identity to the human counterpart, respectively. Complete comparison of percent identity and percent divergence for CD89 of all characterized species is given in Table 3.1. Amino acid differences in the S1 and S2 exons between any of the primates were not identified. There is a higher frequency of CD89 substitutions clustered in the EC1 exon as compared to the overall molecule and EC2 across all phyla for which CD89 has been identified (Tables 3.1-3.3). Figure 3.3 shows a phylogenetic analysis of CD89 from all available species.

Baboon and sooty mangabey CD89 transcripts were variable due to both alternative RNA splicing and sequence substitutions. Several distinct mRNA splice

Figure 3.2. (Next page) Amino acid sequences of baboon and sooty mangabey CD89 aligned with those of other primates. Baboon and sooty mangabey sequences were deduced from experimentally derived sequences of cDNA from whole blood and compared with published sequences from human (GenBank accession number NM_002000), chimpanzee (GenBank BK005386), rhesus macaque (GenBank AY386684) and cynomolgus macaque (GenBank AY386690). The chimpanzee sequence is based on a third party annotation derived from the genome project sequence and has not been confirmed experimentally. Amino acid differences are underlined. The first amino acid of the preprotein is numbered as residue 1. The mature peptide starts at residue 22. Arrows indicate distinct domains. The first two amino acids for EC1 are encoded at the end of the S2 exon. The signal peptide is encoded by both S1 and S2 sequences. Potential N-glycosylation sites, cysteines involved in disulfide bonds and arginine 209 critical for association with the FcR γ chain are bolded. Amino acids that form interface with IgA Fc region are in grey (Herr *et al.*, 2003). Hu: human; Chimp: chimpanzee; Paca: baboon; Soma: sooty mangabey; Mamu: rhesus macaque; Mafa: cynomolgus macaque.

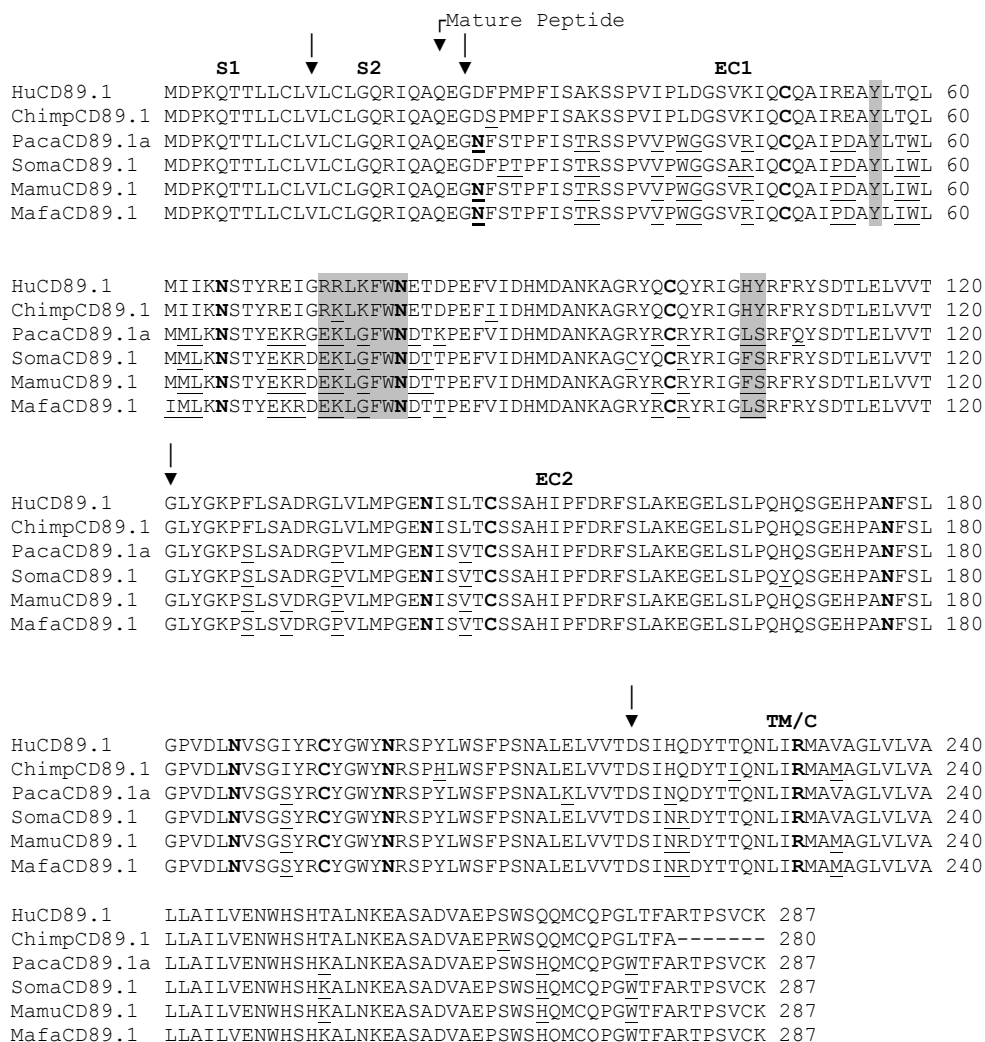


Figure 3.2.

Table 3.1. Percent identities and divergences of CD89 amino acid sequences. Sequences represent all identified CD89 molecules. GenBank accession numbers of previously identified genes are in the text. Paca: baboon; Soma: sooty mangabey; Mamu: rhesus macaque; Mafa: cynomolgus macaque.

		Percent Identity											
		1	2	3	4	5	6	7	8	9	10		
Percent divergence	1		97.5	86.4	87.1	86.4	86.1	55.7	54	55.7	49.6	1	Human
	2	2.5		85.4	85	85	84.6	55.7	53.6	54.9	50.4	2	Chimpanzee
	3	13.8	16.3		94.4	95.1	95.8	54.6	51.7	54.9	48.6	3	Paca
	4	14.2	16.8	4.7		97.2	96.5	53.9	51.6	53.8	49.6	4	Soma
	5	15	16.8	4	2.8		98.6	53.9	52.3	53.8	49.6	5	Mamu
	6	15.5	17.2	3.2	3.6	1.4		53.5	51.9	54.2	49.3	6	Mafa
	7	64.1	63.3	65.8	67.5	67.5	68.3		69	67.4	55	7	Horse
	8	66.4	67.3	70.9	72.2	70.5	71.4	39.9		70.8	50.7	8	Cow
	9	60.7	61.5	62.4	64.9	64.9	64.1	40.4	35.1		55.3	9	Pig
	10	69.8	67.4	71.1	69	69	69.8	60.2	69	60.2		10	Rat
		1	2	3	4	5	6	7	8	9	10		

Table 3.2. Percent identities and divergences for deduced amino acid sequences of CD89 extracellular domain 1, the IgA binding domain. GenBank accession numbers of previously identified genes are in the text. Paca: baboon; Soma: sooty mangabey; Mamu: rhesus macaque; Mafa: cynomolgus macaque.

CD89 EC1

		Percent Identity											
		1	2	3	4	5	6	7	8	9	10		
Percent Divergence	1		96.9	72.2	72.2	72.2	70.1	46.3	49.5	47.4	42.6	1	Human
	2	3.2		71.1	71.1	71.1	69.1	49.5	50.5	48.4	43.6	2	Chimpanzee
	3	34.8	36.4		89.7	93.8	94.8	42.1	46.3	48.4	41.5	3	Paca
	4	34.8	36.4	11.1		95.9	92.8	43.2	45.3	46.3	44.7	4	Soma
	5	34.8	36.4	6.5	4.2		96.9	43.2	47.4	47.4	42.6	5	Mamu
	6	38.1	39.8	5.3	7.6	3.2		42.1	46.3	48.4	41.5	6	Mafa
	7	84.2	75.7	93.5	90.3	90.3	93.5		74.7	72.6	59.6	7	Horse
	8	73.1	70.5	81.3	84.2	78.5	81.3	30.8		72.6	55.3	8	Cow
	9	78.5	75.7	75.7	81.3	78.5	75.7	34.1	34.1		59.6	9	Pig
	10	98.7	95.3	95.3	88.8	92	95.3	59.7	66.7	57.4		10	Rat
		1	2	3	4	5	6	7	8	9	10		

Table 3.3. Percent identities and divergences for deduced amino acid sequences of CD89 extracellular domain 2. GenBank accession numbers of previously identified genes are in the text. Paca: baboon; Soma: sooty mangabey; Mamu: rhesus macaque; Mafa: cynomolgus macaque.

CD89 EC2

Percent Identity

	1	2	3	4	5	6	7	8	9	10			
Percent Divergence	1		99	94.8	94.8	94.8	61.5	61.5	65.6	65.6	1	Human	
	2	1		93.8	93.8	93.8	60.4	60.4	64.6	64.6	2	Chimpanzee	
	3	5.4	6.5		97.9	97.9	97.9	60.4	59.4	61.5	63.5	3	Paca
	4	5.4	6.5	2.1		97.9	97.9	58.3	59.4	61.5	63.5	4	Soma
	5	5.4	6.5	2.1	2.1		100	59.4	60.4	61.5	64.6	5	Mamu
	6	5.4	6.5	2.1	2.1	0		59.4	60.4	61.5	64.6	6	Mafa
	7	53.6	55.7	55.7	60	57.8	57.8		65.6	66.7	61.5	7	Horse
	8	53.6	55.7	57.8	57.8	55.7	55.7	45.8		75	61.5	8	Cow
	9	45.8	47.7	53.6	53.6	53.6	53.6	43.9	30.4		63.5	9	Pig
	10	45.8	47.7	49.6	49.6	47.7	47.7	53.6	53.6	49.6		10	Rat
	1	2	3	4	5	6	7	8	9	10			

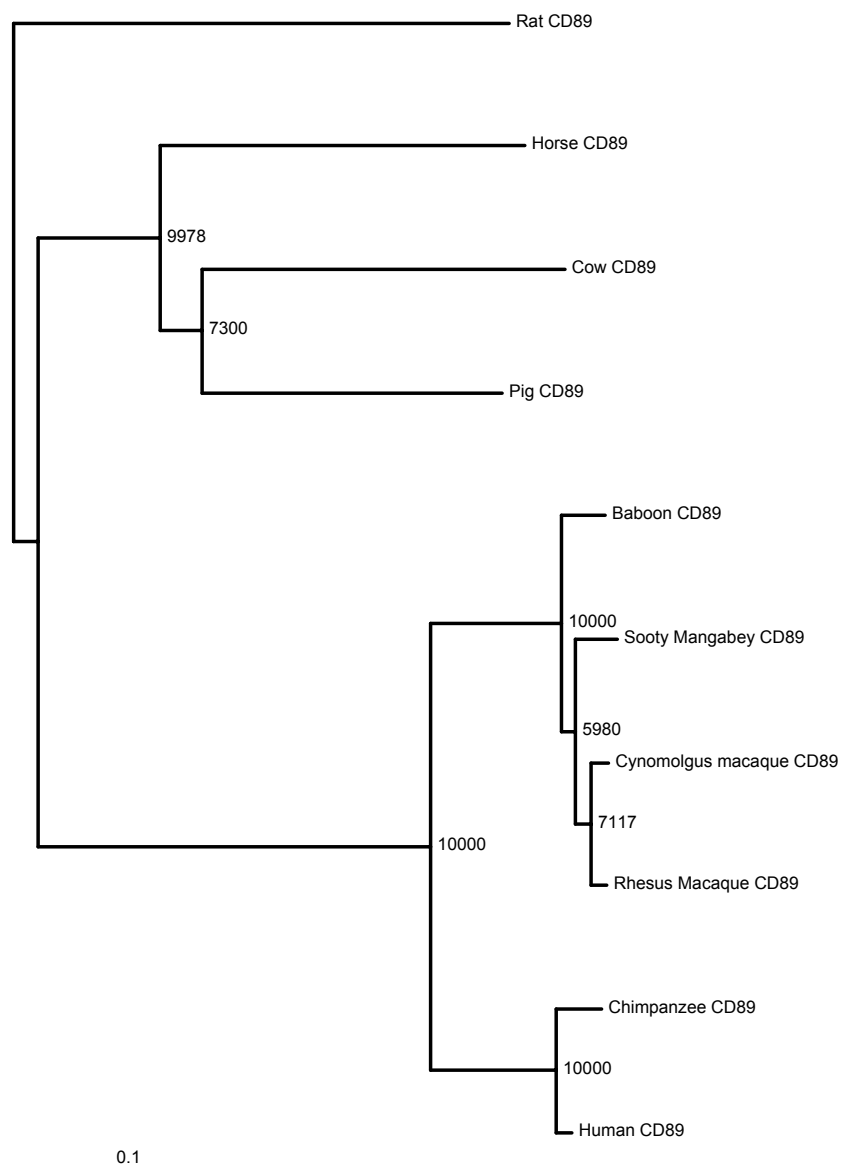


Figure 3.3. Neighbor joining (NJ) phylogenetic tree constructed from the amino acid sequences of all identified CD89 molecules in various species. Bootstrapping of the NJ tree was performed with 10,000 replicates. Baboon and sooty mangabey sequences are derived from this study, GenBank accession numbers for the remaining species are given in the Materials and Methods section. The bar indicates the number of substitution per site.

variants CD89 have been identified in humans and macaques, but few splice variants have been identified in other species (Morton *et al.*, 1996; Patry *et al.*, 1996; Pleass *et al.*, 1996; Reterink *et al.*, 1996; Maruoka, 2004; Morton *et al.*, 2004; Rogers *et al.*, 2004; Morton *et al.*, 2005). As was the case in other primates, we identified several splice variants in baboons and in mangabeys. We identified eight splice variants for four different baboons and three splice variants from a single sooty mangabey in addition to the full length transcript. Figures 3.4 and 3.5 depict schematically the splice variants identified for the baboons and the sooty mangabey, respectively. For baboons, five of the eight splice variants (PacaCD89.3, PacaCD89.4, PacaCD89.7, PacaCD89.9 and PacaCD89 Δ EC1 Δ EC2) are similar to described human CD89 isoforms (Monteiro and van de Winkel, 2003; Pleass *et al.*, 1997), whereas three of the variants (PacaCD89 Δ EC1, PacaCD89 Δ EC1 Δ TM/C and PacaCD89 Δ S2 Δ EC1 Δ TM/C) have not been described in humans. Two sooty mangabey splice variants are similar to reported human splice variants (SomaCD89.3 and SomaCD89.9). The third sooty mangabey variant has not been described in humans (SomaCD89 Δ S2 Δ EC1 Δ TM/C).

We have previously noted a high degree of polymorphism of the CD89 ligand, IgA, for nonhuman primates and have considered the possibility that gene conversion may account for some of this variation. To determine whether or not this IgA diversity might correspond to polymorphisms in CD89, we examined CD89 from several baboons.

Genetic variations resulting in amino acid substitutions were identified in three of the baboons examined and in the sooty mangabey. Two nucleotide sequences that differed only by a single silent substitution were obtained from the fourth baboon. The deduced

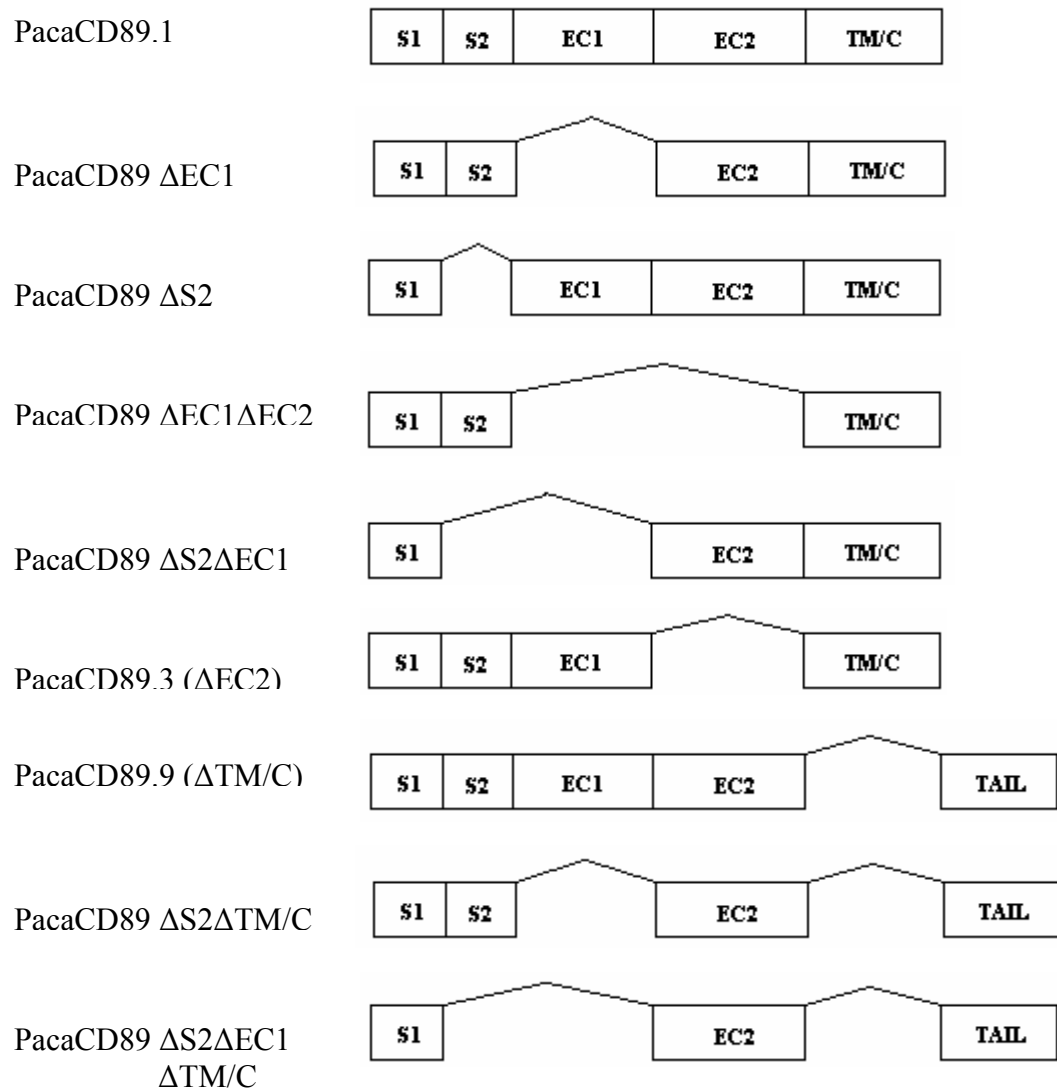


Figure 3.4. Schematic representation of the full-length CD89 transcript and corresponding splice variants identified in baboons. Paca: *Papio hamadryas anubis*.

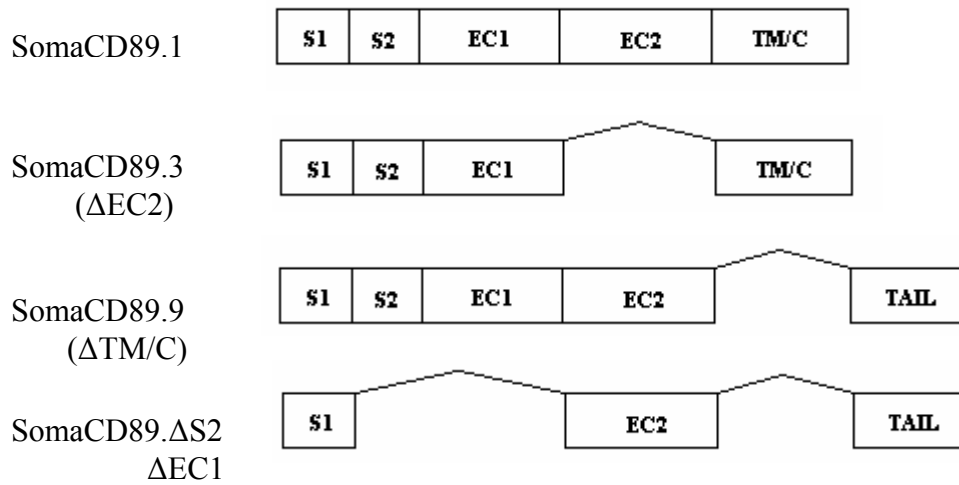


Figure 3.5. Schematic representation of the full-length CD89 transcript and corresponding splice variants identified in sooty mangabey. Soma: Sooty mangabey.

amino acid sequences of the different sequence variants of the CD89 transcripts and splice variants of each of the four baboons and of the sooty mangabey are shown in Figures 3.6-3.10. CD89 gene sequence variations were studied further in baboons using two pedigreed families consisting of a sire, a dam and four offspring. All of the different deduced amino acid sequences for baboon CD89 representing sixteen animals examined are presented in Figure 3.11. The offsprings' CD89 genes were inherited with the expected patterns of normal Mendelian genetics (Figure 3.12).

Expression of CD89 on baboon and sooty mangabey blood leukocytes

In humans and macaques, CD89 is expressed only on select cells of the myeloid lineage and not on lymphocytes. To determine whether or not baboon and sooty mangabey CD89 is similarly expressed, whole blood from four animals of each species was stained with the anti-human CD89 PE and anti-human CD16 FITC (sooty mangabeys only) and analyzed by flow cytometry. Lymphocytes, monocytes, and granulocytes (neutrophils and eosinophils) were identified using forward and side scatter properties and each population gated. For all four baboons examined, no well delineated population of monocytes could be identified. These samples must be shipped overnight, and our results possibly reflect a selective loss of this population as the blood ages. By contrast, mangabey blood which was only two to four hours old when processed had well defined monocyte populations. Mangabeys expressed CD89 on $72.61 \pm 12.25\%$ (mean \pm standard deviation) of their total leukocytes with no appreciable expression on lymphocytes ($0.64 \pm 0.17\%$) and expression on a majority of monocytes and granulocytes

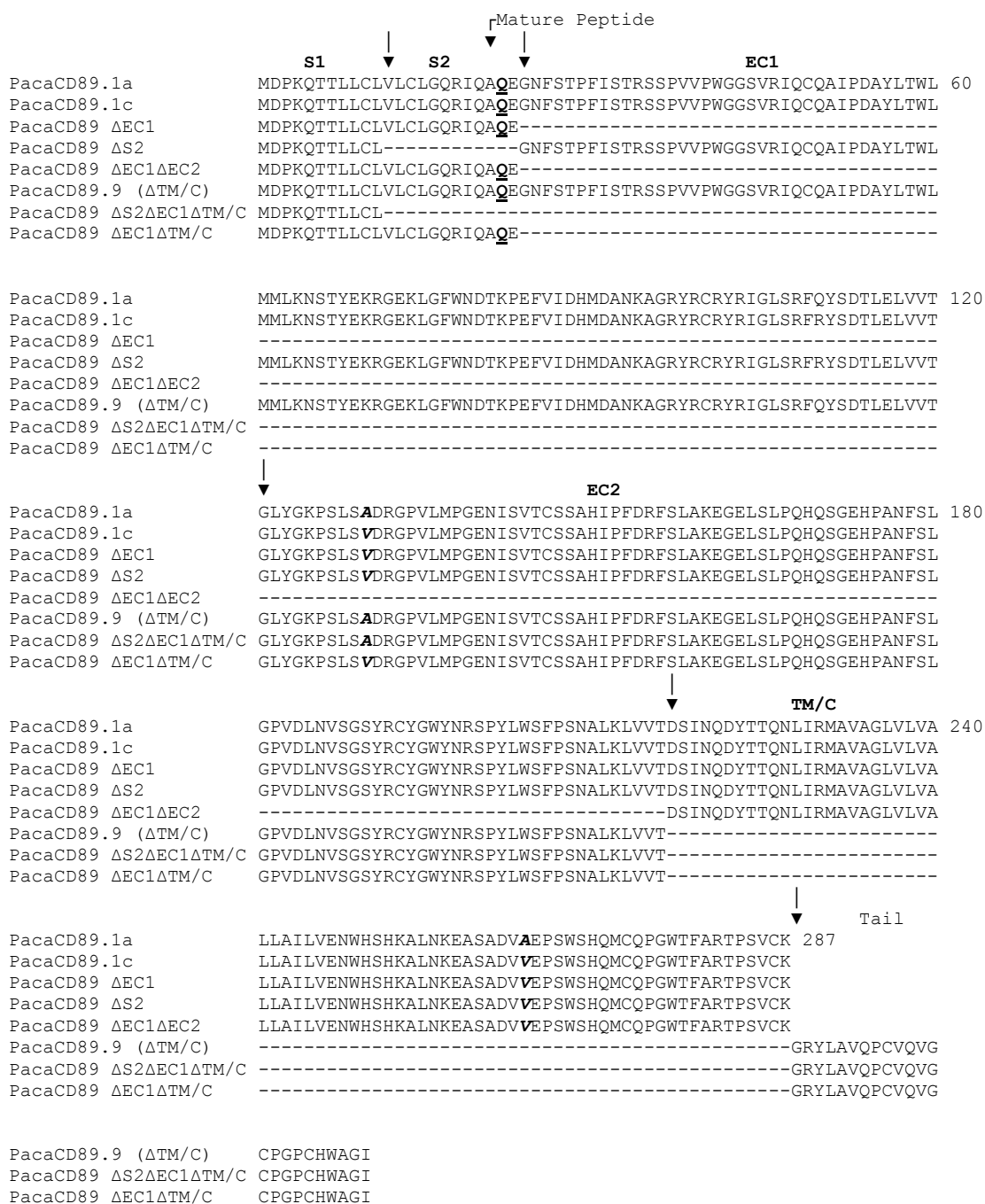


Figure 3.6. Alignment of baboon CD89 splice variants and polymorphisms from animal #35. Amino acid differences between sequences are bolded and italicized. Paca: *Papio hamadryas anubis*.

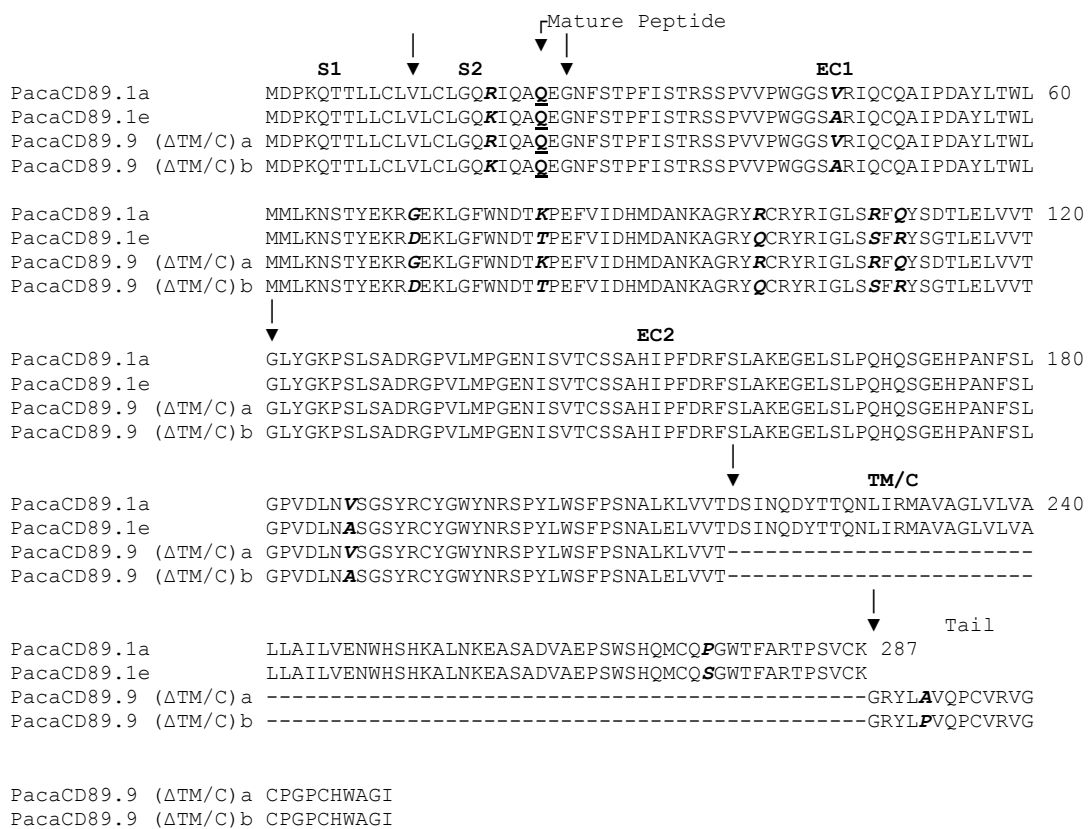


Figure 3.7. Alignment of baboon CD89 splice variants and polymorphisms from animal #36. Amino acid differences between sequences are bolded and italicized. Paca: *Papio hamadryas anubis*.

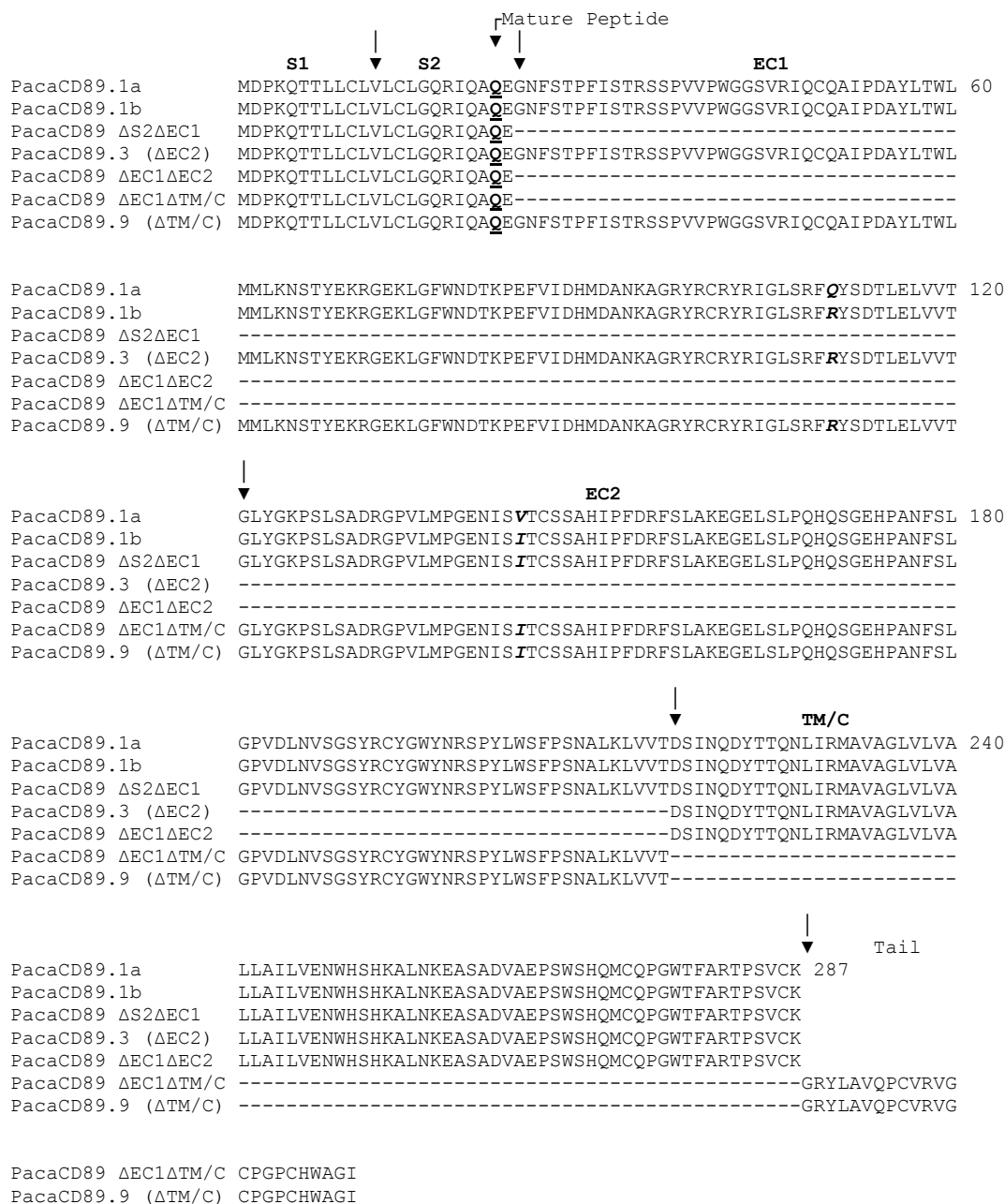


Figure 3.8. Alignment of baboon CD89 splice variants and polymorphisms from animal #38. Amino acid differences between sequences are bolded and italicized. Paca: *Papio hamadryas anubis*.

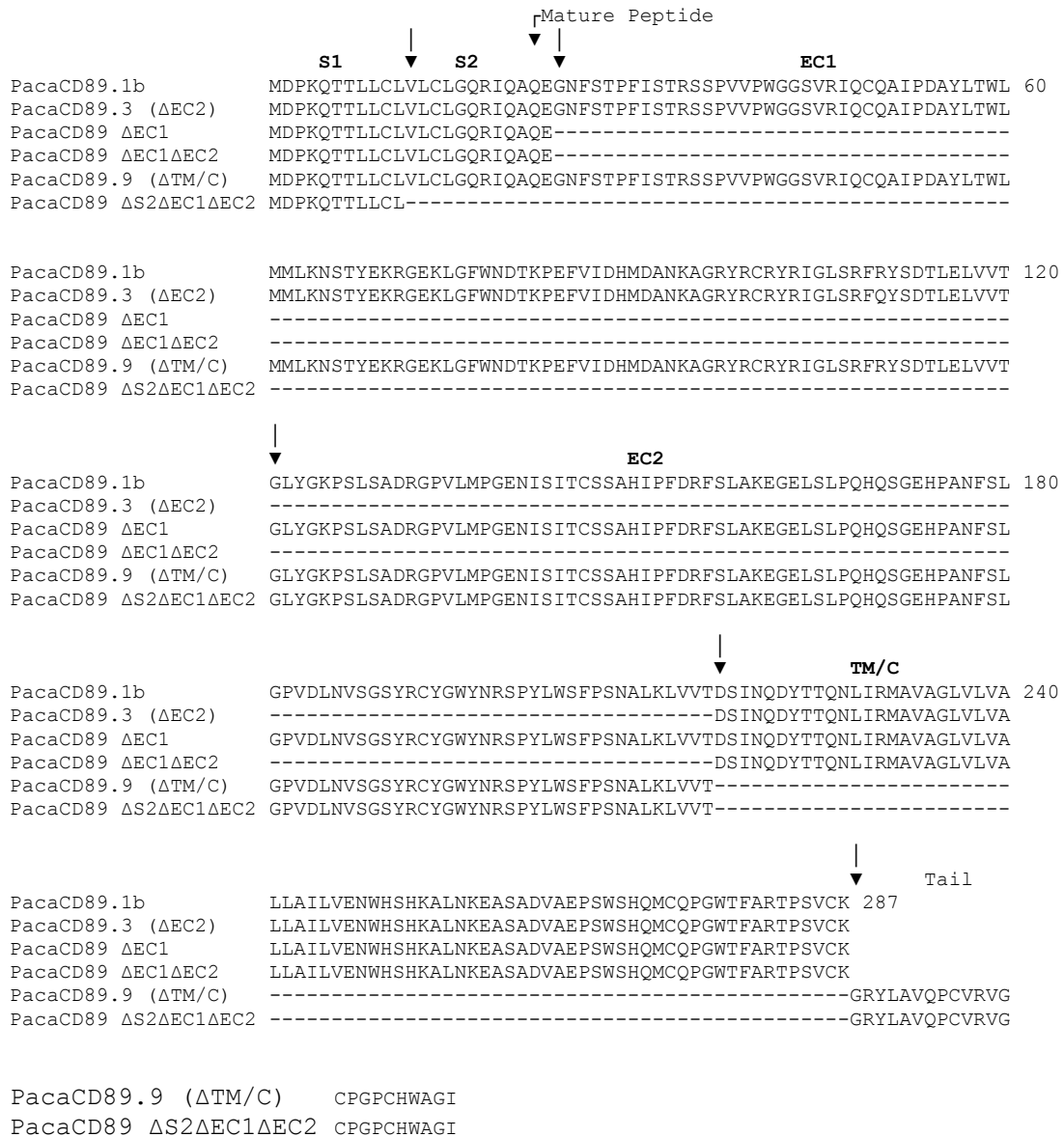


Figure 3.9. Alignment of baboon CD89 splice variants and polymorphisms from animal #39. Amino acid differences between sequences are bolded and italicized. Paca: *Papio hamadryas anubis*.

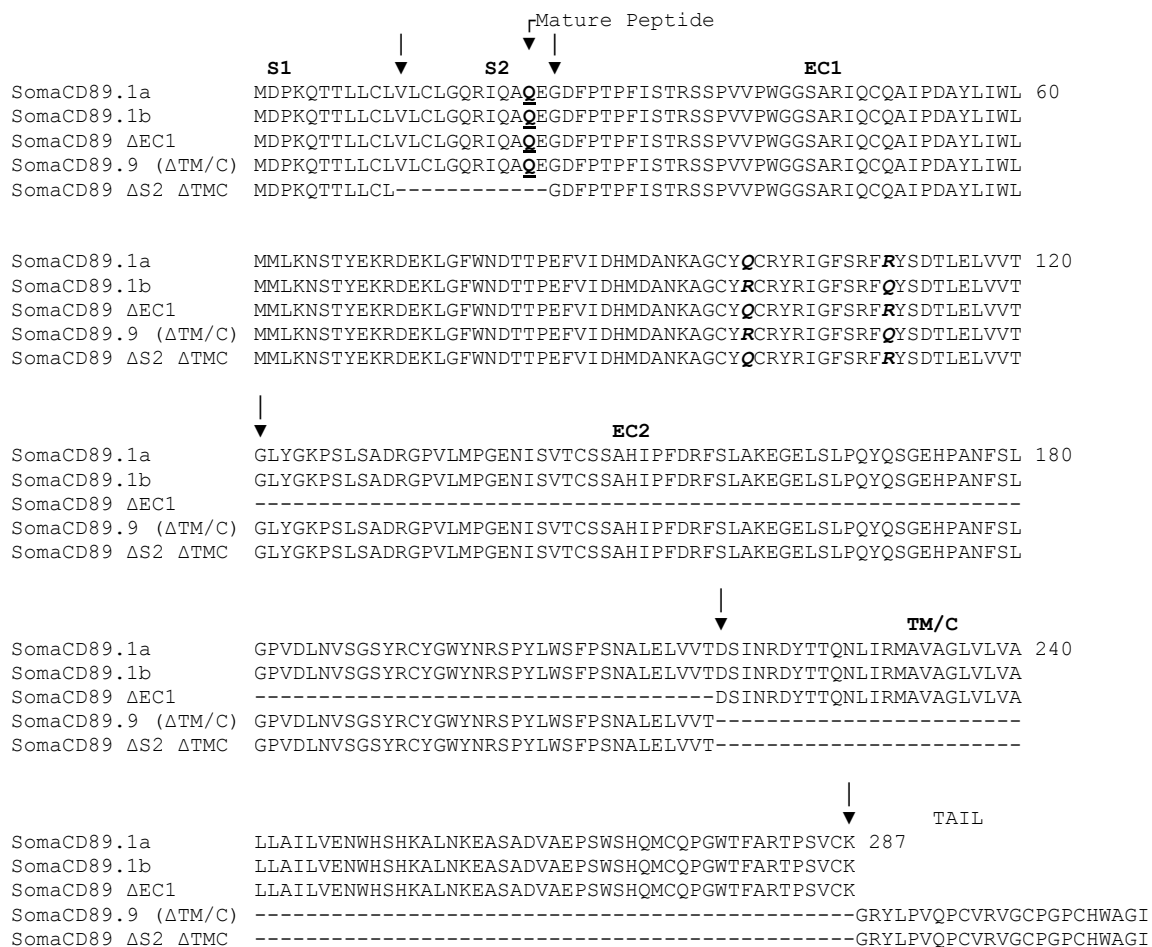


Figure 3.10. Sooty mangabey CD89 splice variant alignment and amino acid

substitutions. Amino acid differences between sequences are bolded and italicized. Soma: sooty mangabey.

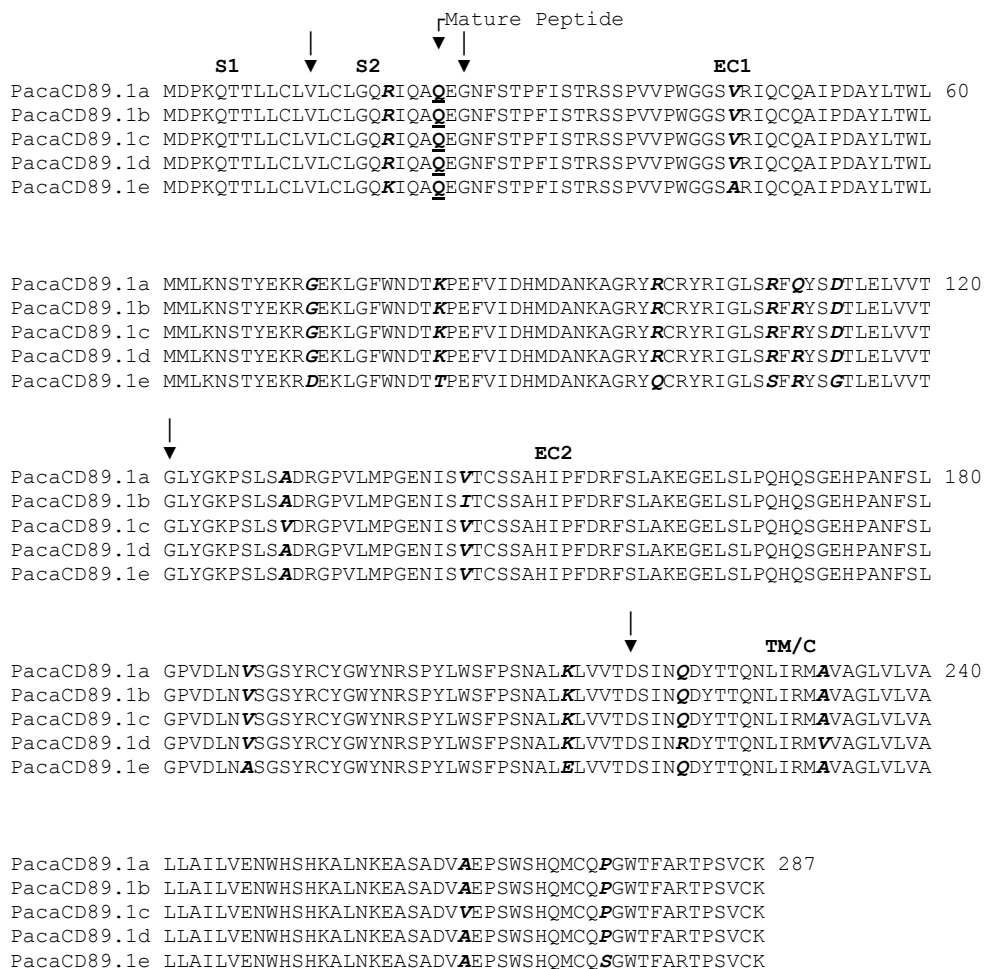


Figure 3.11. Baboon CD89 polymorphisms. Positions that are different between sequences are bolded and italicized. Form “a” is found in baboons 35, 36, 38, 7808, 8344, 8170, and 10595 albeit there are differences in some monkeys in the nucleic acid sequence. Form “b” is found in baboons 39 (two different nucleic acid sequences) and in baboon 38. The remaining three peptide sequences are deduced from a single baboon: “c” for baboon 35, “d” for baboon 7808, “e” for baboon 36.

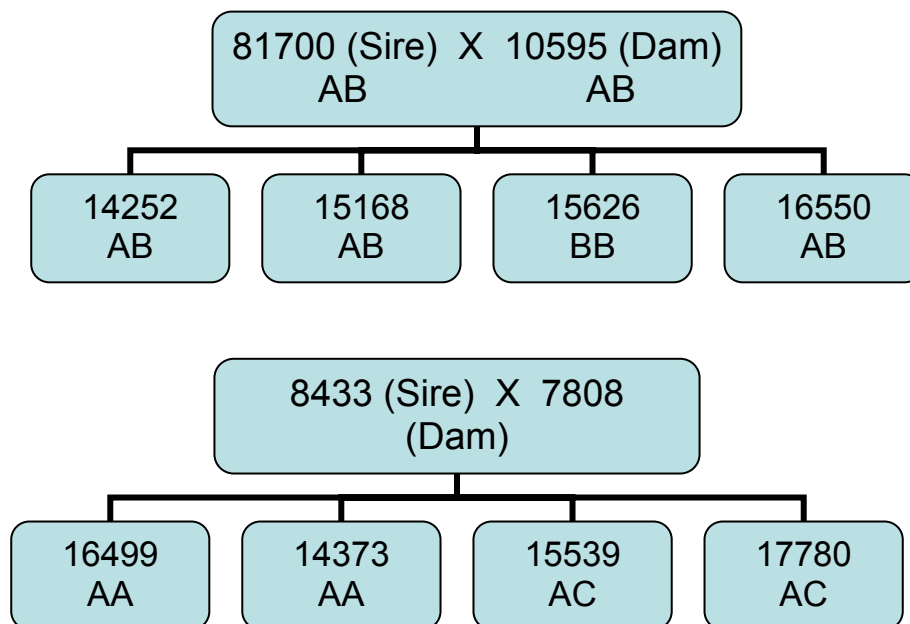


Figure 3.12. Inheritance of CD89 gene sequence variants among baboons in two pedigreed baboon families. Diagrams above give the baboon numeric designation assigned by the Southwest National Primate Research Center above the genotypes of each monkey. Genes are arbitrarily designated A, B and C. Genes A and B differ by a single nucleotide and do not differ in the amino acid sequence and match the PacaCD89.1a amino acid sequence of figure 3.11. Gene C encodes a protein matching PacaCD89.1e of figure 3.11.

(83.45±6.08% and 99.30±0.58% CD89⁺ respectively) (Figure 3.13). CD89 staining of mangabey cells was specific, since an irrelevant isotype matched PE labeled mouse antibody did not bind to the cells (CD89⁺ 0.37±0.63). CD16, the IgG low affinity Fc receptor III, was coexpressed on a majority of mangabey granulocytes with CD89, but was only expressed on a minority of cells in the monocyte gate, a few of which did not express CD89. Baboons expressed CD89 on their granulocytes (mean fluorescence intensity (MFI) = 162.16±37.98), but not on lymphocytes (MFI = 5.58±1.68) (Figure 3.14). A mouse isotype control did not bind to baboon leukocytes (MFI = 7.31±2.96).

Expression of recombinant rhesus macaque CD89 in HeLa cells, IgA binding and effects of glycosylation inhibition

HeLa cells were transfected with an expression vector for full-length rhesus macaque CD89 designed to incorporate itself in chromosomes for stable expression. After antibiotic selection, isolation, expansion and screening, three clones positive for CD89 cDNA transcripts and exhibiting high CD89⁺ fluorescence staining were identified (Figure 3.15). Cells from the positive clones were incubated with mouse isotype control PE and untransfected HeLa cells incubated with anti-human CD89 PE. All these cells were negative for fluorescence staining, thus verifying that CD89⁺ staining of these clones was specific. Expression of CD89 from these cells was stable, since CD89 expression was consistently detected on these cells for over one year.

Using clones expressing CD89 and untransfected control HeLa cells, binding of heat aggregated human myeloma proteins and recombinant chimeric rhesus IgA to rhesus macaque CD89 was assessed (Figures 3.16 and 3.17). Cells were simultaneously stained

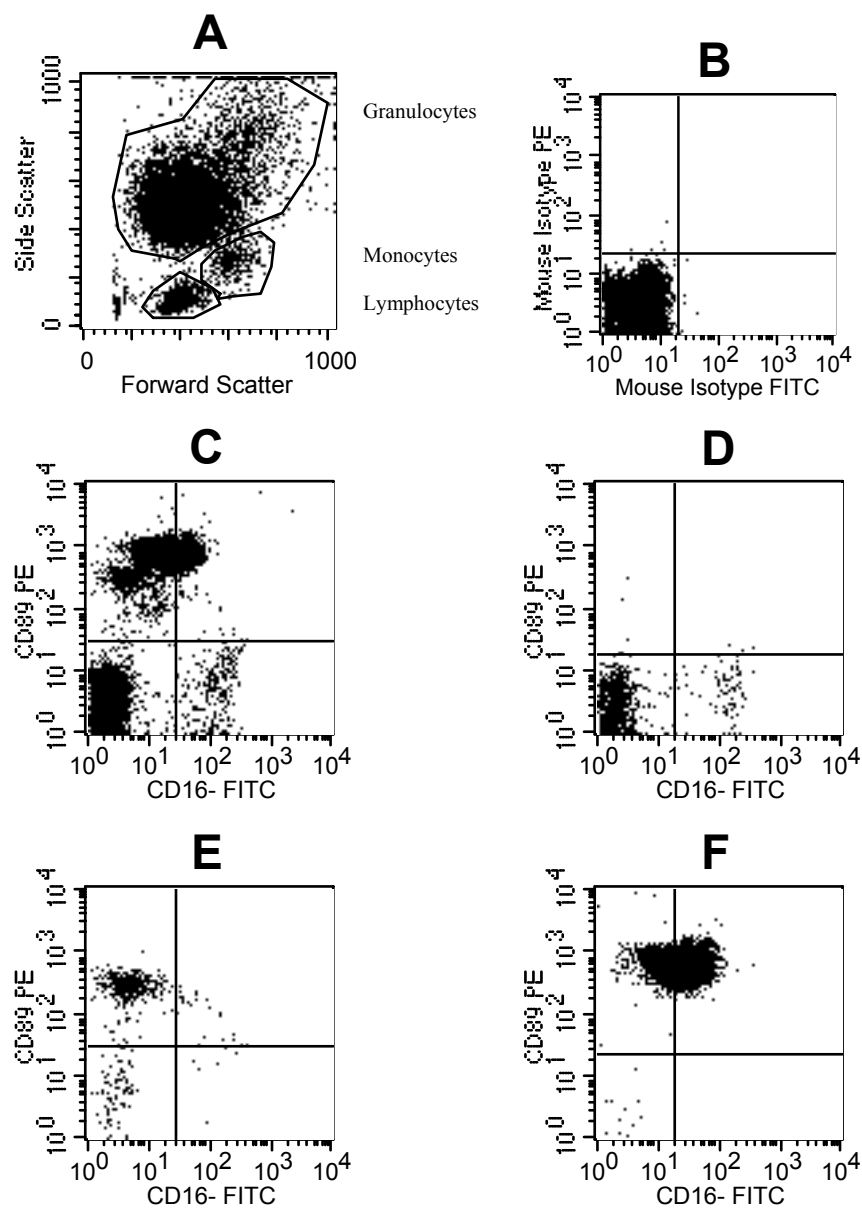


Figure 3.13. Sooty mangabey CD89 is expressed on monocytes and granulocytes. Two-color dot-plots of whole blood leukocytes from a representative sooty mangabey showing cell population expressing CD89. Forward scatter versus side scatter plot (A); mouse isotype control PE versus mouse isotype control FITC (B); CD16 versus CD89 for all leucocytes (C), gated lymphocytes (D), gated monocytes (E), and gated granulocytes (F).

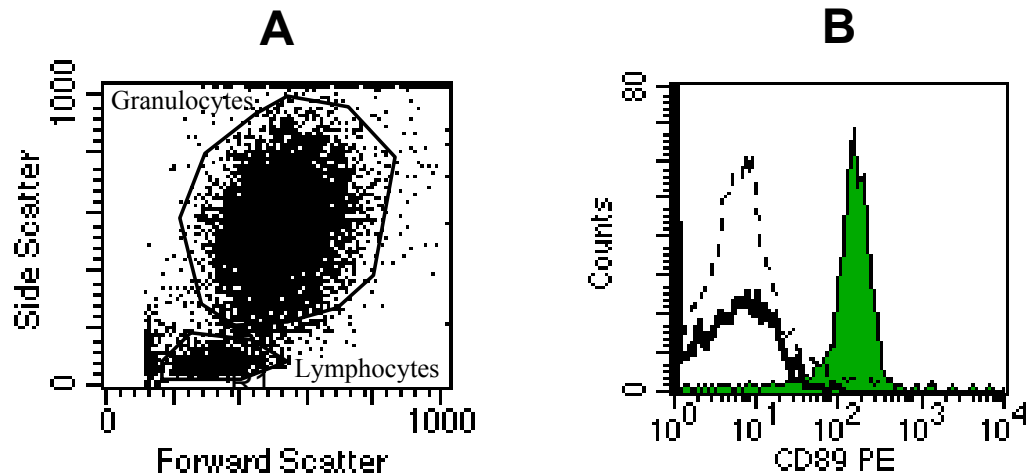


Figure 3.14. Baboons express CD89 on granulocytes, but not on lymphocytes from whole blood. Forward scatter versus side scatter dot-plot (A) showing gated lymphocytes and granulocytes (no defined monocyte populations could be identified for any of the baboon samples stained for CD89). Histogram of baboon whole blood leukocytes staining for CD89 (B): green curve = granulocytes, thick line = lymphocytes, dotted line = total leukocytes stained with PE labeled mouse isotype control.

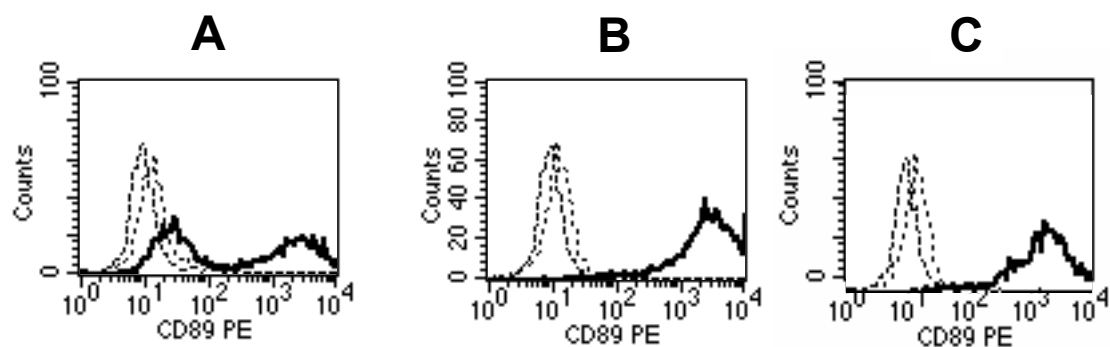


Figure 3.15. Expression of recombinant rhesus macaque CD89 on HeLa cells. (A) Non-clonal HeLa cells expressing CD89 and (B and C) HeLa cell clones expressing CD89. HeLa cells were stained with anti-human CD89 PE and 5000 counted by flow cytometry. Thick solid line = transfected cells, dashed line = untransfected HeLa cells, dotted line = transfected cells strained with an isotype control.

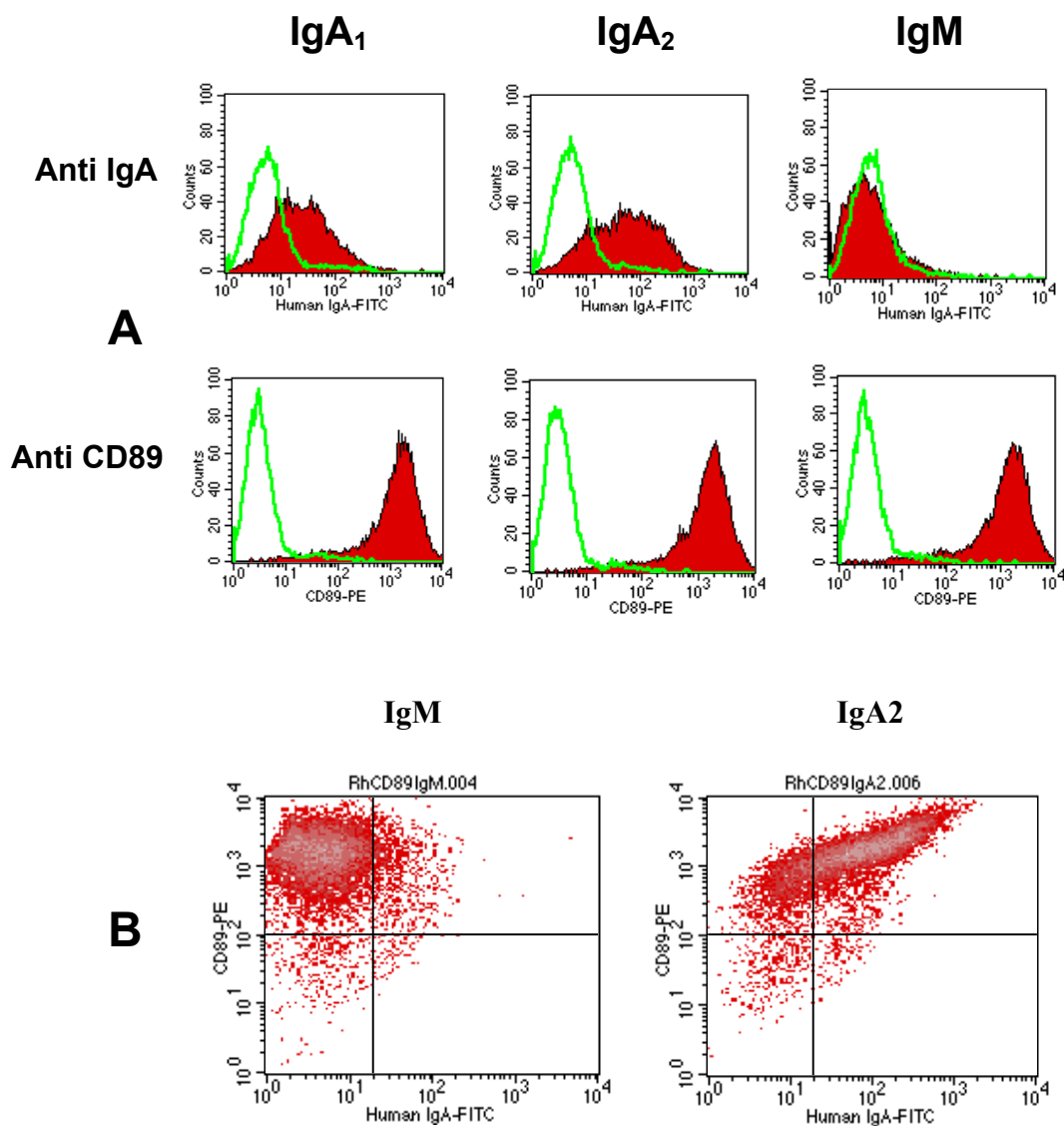


Figure 3.16. Recombinant rhesus macaque CD89 binds to heat aggregated human myeloma IgA1 and IgA2 (representative experiment). A: histogram plots of HeLa cells expressing rhesus macaque CD89 (filled red curves) or control cells (green line) stained with anti-human CD89 PE and anti-human IgA FITC. B: two-color dot plots of anti-human IgA FITC versus anti-human CD89 PE. 10,000 cells were counted for each assay.

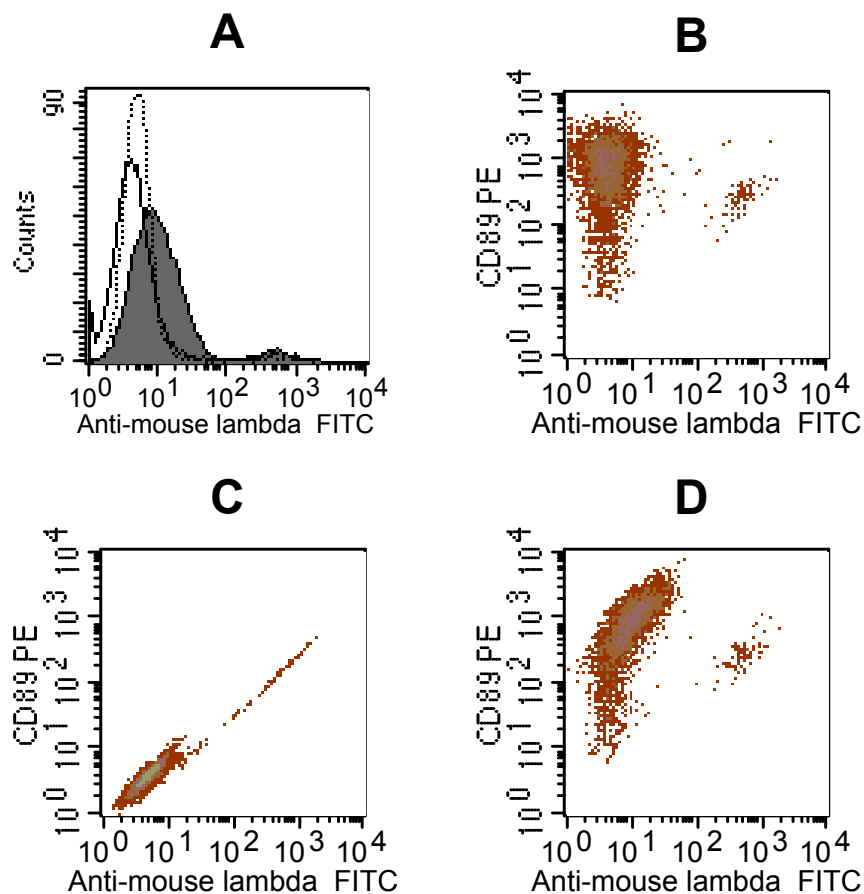


Figure 3.17. Rhesus IgA binds to rhesus macaque CD89. HeLa cells expressing recombinant rhesus macaque CD89 were incubated with supernatant of J588L cells expressing recombinant rhesus IgA (containing a mouse Ig λ) or control supernatant and stained with anti-mouse Ig λ FITC and anti-human CD89 PE. A: histogram plot of the HeLa cells expressing CD89 incubated with rhesus IgA (filled), or control supernatant (dashed line), and untransfected control HeLa cells incubated with IgA (thick line). Two-color dot plots (C) HeLa cells with IgA supernatant, (B) and (D) HeLa cells expressing CD89 cells with control supernatant and IgA supernatant, respectively.

with anti-human CD89 PE and either anti-human Ig κ or anti-human Ig α to detect bound antibodies. Human IgA1 and IgA2, but not human IgG or IgM, bound to CD89 (Figure 3.16). IgA binding increased with increased expression of CD89 as indicated by the positive correlation of MFI for CD89 staining plotted against MFI for bound IgA staining (Figure 3.16). No correlation exists for MFI of CD89 staining plotted against MFI of staining for isotypes that did not bind IgM or IgG (Figure 3.16). Multiple experiments were carried out to test for CD89 binding to NIP specific rhesus IgA. The presence of recombinant IgA in the supernatant of transfected J558L cells was verified by ELISA (data not shown). When rhesus IgA binding was tested in the presence of the antigen NIP (5) BSA, staining against the recombinant antibody was positive for CD89 cells incubated with IgA supernatant (10.81 ± 8.15), but not for HeLa cells (5.45 ± 3.37) or CD89 cells incubated with control supernatant (5.00 ± 4.61) (Figure 3.17). Thus, binding of rhesus macaque IgA to rhesus macaque CD89 was detectable, although IgA was present at much lower concentrations in the supernatant when compared to the concentration of the human IgA myeloma protein in its corresponding binding assay.

To assess whether or not rhesus IgA interactions with CD89 were dependent on IgA binding to antigen, a variation of the binding assay was performed by titrating out the concentration of NIP (5) BSA incubated with the supernatants (Table 3.4). Binding to IgA was detectable in the absence of antigen, increased slightly with antigen concentrations of 1.88-7.5 μ g/ml and declined with further excess of antigen (15 μ l/ml). MFI for staining of bound IgA was significantly less for both controls using untransfected HeLa cells incubated with IgA supernatant ($p = 0.005$) and HeLa cells

Table 3.4. Binding of rhesus macaque CD89 to recombinant rhesus IgA incubated with different concentrations of antigen, NIP (5) BSA. HeLa cells expressing recombinant rhesus macaque CD89 or untransfected control HeLa cells were incubated with supernatant of J588L cells expressing recombinant rhesus IgA (containing a mouse Ig λ) or control supernatant which contained the indicated concentrations of NIP (5) BSA and stained with anti-mouse Ig λ FITC to detect bound rhesus IgA. MFI: mean fluorescence intensity.

NIP (5) BSA (μ g/ml)	Rhesus IgA & Rhesus Macaque CD89 (MFI)	Control Supernatant & Rhesus Macaque CD89 (MFI)	Rhesus IgA & HeLa Cells (MFI)
0	27.6	21.8	22.8
1.88	31.6	20.2	25.7
3.75	28.9	24.3	18.2
7.5	31.3	24.3	18.7
15	26.6	21.4	19.0
Mean \pm Std	29.2 \pm 2.2	22.4 \pm 1.8	20.9 \pm 3.3

expressing CD89 incubated with control supernatant ($p = 0.005$), but not between the two control groups ($p = 0.52$) as determined using a paired student T-test.

Fc receptor glycans are often important for expression and immunoglobulin Fc binding (Drescher *et al.*, 2003). We examined the effects of blocking N-glycosylation with tunicamycin on rhesus CD89 expression and binding properties, since rhesus macaque CD89 has N-glycosylation motifs conserved with sites in human CD89 occupied by N-glycans (Figure 3.18). Expression of CD89 was dramatically reduced in cells treated with tunicamycin (57%-73% reduction) with a disproportionately greater decrease in ability to bind to human IgA1 and IgA2 (84%-95%).

Splice variants of rhesus macaque CD89

Attempts were made to isolate clones with high expression levels of two rhesus macaque splice variants: CD89 Δ EC1, which has the first extracellular domain deleted, and CD89.9 (CD89 Δ TM/C), which has the transmembrane and cytoplasmic domains deleted. Despite isolating clones for each variant that produced transcripts, none of the clones stained for high levels of CD89 expression. The monoclonal antibody used to detect CD89 expression (A59) binds to the EC2 domain, which is present in both transcripts; therefore, failure to detect the CD89 isoforms is unlikely to be due to the absence of the binding epitope. There was some evidence for low level expression of the resulting isoforms for each of these splice variants. For some clones, low expression levels were detected by flow cytometry on the cell surface (Figure 3.19).

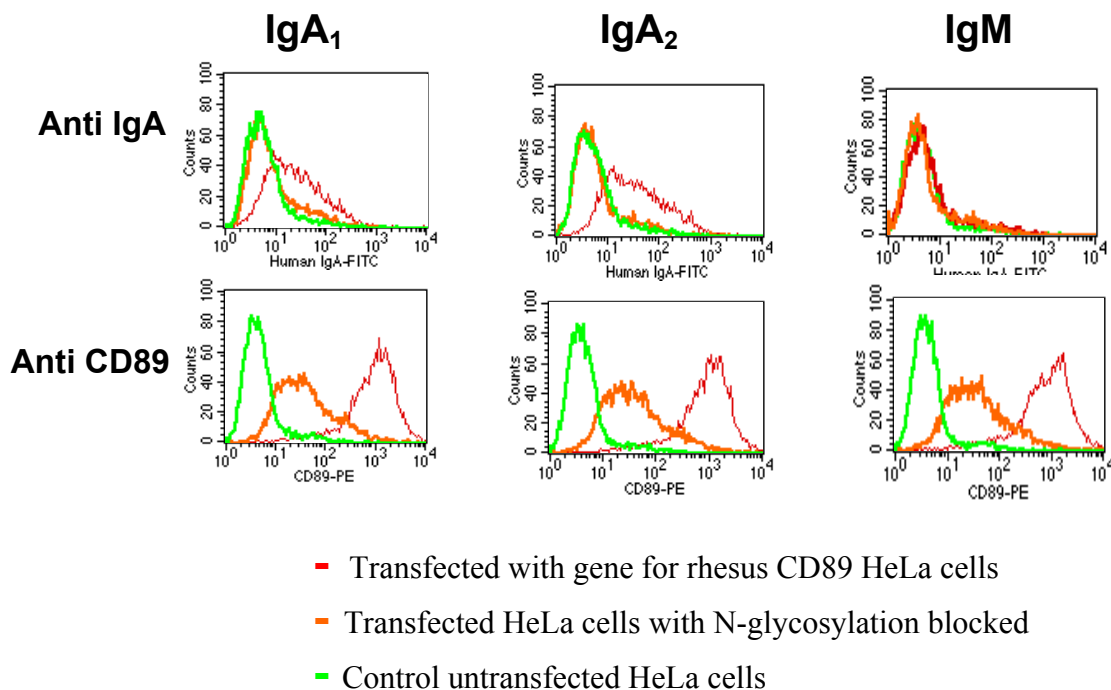


Figure 3.18. Blocking N-glycosylation reduces expression of recombinant rhesus macaque CD89 and results in a strong decrease in binding of human IgA1 and IgA2. HeLa cells were treated with tunicamycin to inhibit the N-glycosylation pathway.

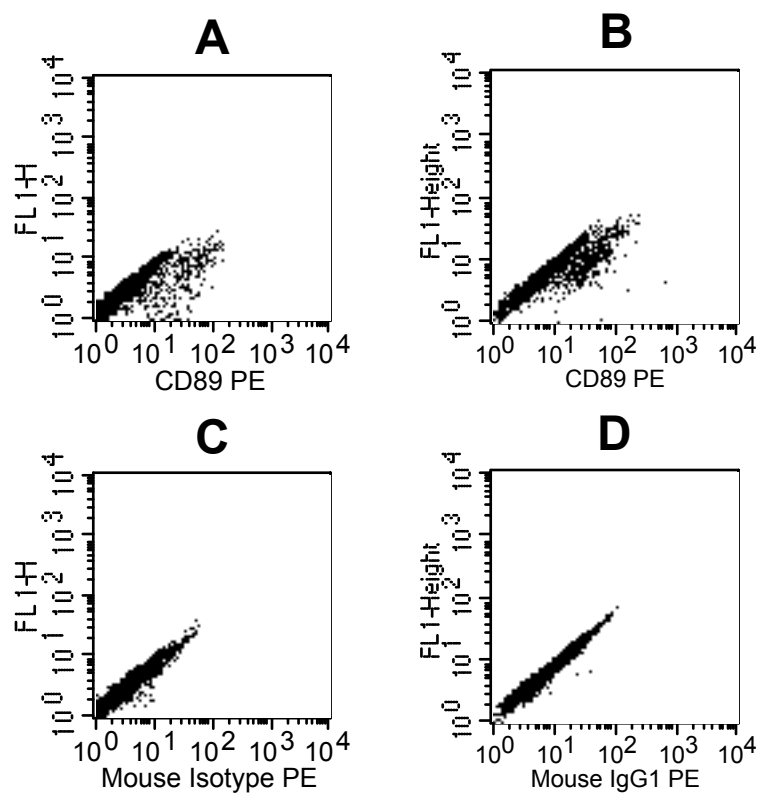


Figure 3.19. Rhesus CD89 isoforms expressed at low levels in HeLa cells. Two-color dot plot showing specific staining against CD89 (A, B) and the mouse isotype control (C, D). Clones for the expression of CD89 Δ EC1 (A, C), and of CD89 Δ TM/C (B, D) are shown from representative clones. MFI of CD89 staining is indicated on the X-axis. MFI of auto-fluorescence is indicated on the Y-axis indicates.

While some recombinant human CD89.9 is expressed on the surface of Cos-7 cells, most of the product is secreted into the media (van Dijk, 1996). Therefore, attempts were made to detect rhesus macaque CD89.9 in the media. Secreted CD89.9 was not detected by ELISA, but no positive control was available for these experiments (data not shown). Since CD89.9 was difficult to detect directly, transfected cells were tested for its expression indirectly by assaying for IgA binding. MFI did not convincingly increase when cells were stained for bound IgA, but an increase of MFI for CD89 staining of these cells was observed after incubation with IgA. Therefore, it was hypothesized that cells expressed low levels of CD89.9 that could bind to HA IgA complexes, which in turn allowed binding of soluble CD89.9 to unoccupied IgA binding sites. This would result in an increased concentration of CD89.9 associated with the cells, and hence the observed increase in MFI for CD89 staining. To test this hypothesis, a similar assay was performed. HeLa cells expressing full length CD89 were incubated with human IgA and either media from untransfected HeLa cells or media from cells transfected with the expression vector for CD89.9. In multiple experiments, greater staining for CD89 was observed for cells incubated with CD89.9 media compared to cells incubated with control media (Figure 3.20 A and B). A small but significant decrease in staining for bound IgA was also observed in some experiments under similar circumstances (Figure 3.20 C and D). No differences in staining for CD89 were present between CD89.9 media and control media treated cells when IgM was incubated with cells instead of IgA.

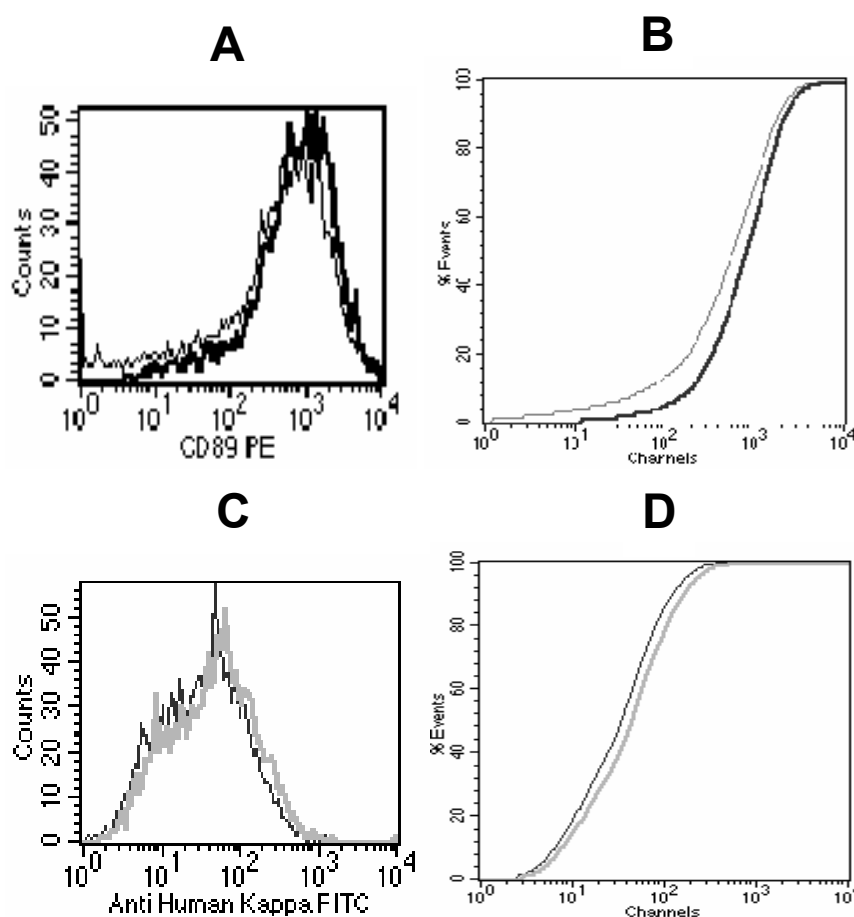


Figure 3.20. Soluble rhesus CD89.9 binds to IgA captured by CD89 on cells and reduces IgA binding. Incubation of media from cells putatively secreting CD89.9 (black line) with HeLa cells expressing rhesus CD89 on the plasma membrane results in an increase in CD89 staining mean fluorescence intensity (MFI) (A, B) and decreased bound IgA staining MFI (C, D) compared to incubation of media from control HeLa cells which do not secrete a CD89 isoform (grey line). A, C: histogram plots; B, D: cumulative fraction plots. Differences were determined to be statistically significant using the Kolmogorov-Smirnov test ($p \leq 0.001$). 10000 cells were counted by flow cytometry for each group.

Discussion

IgA forms an important defense against pathogens at the mucosal surfaces and is found at significant concentration in sera where it can interact with CD89 to engage immune effector functions. IgA/CD89 interactions are important for immune responses to pathogens including mucosal infections with *S. pneumoniae* and *Bordetella pertussis* (Monteiro and van de Winkel, 2003). CD89 plays a protective role in periodontal disease and has been shown to mediate the clearance of *Porphyomonas gingivalis* (Yuan *et al.*, 2000; Kobayashi *et al.*, 2001, 2003). Interestingly, a polymorphism of CD89 involving a silent nucleotide substitution has been shown to be associated with aggressive periodontitis (Kaneko *et al.*, 2003). By contrast, single nucleotide polymorphisms of CD89 do not appear to be risk factors for allergic asthma (Jasek *et al.*, 2004). CD89 function, concentrations in sera as well as on cells, and glycosylation patterns are altered in several diseases including HIV-1 infections (Grossetête *et al.*, 1994), alcoholic cirrhosis (Silvain *et al.*, 1995), ankylosing spondylitis (Montengro *et al.*, 2000) and Henoch-Schonlein purpura (Haddad *et al.*, 2003). Results from preliminary studies show that bispecific therapeutic antibodies and other therapeutics targeting CD89 are potentially efficacious in the treatment of bacterial and fungal infections (Kobayashi *et al.*, 2004; Tacke *et al.*, 2004) and cancers (Valerius *et al.*, 1997; Stockmeyer 2000; Sundarapandiyan 2001).

Several studies have examined a possible role for CD89/IgA interactions in the pathogenesis of IgA nephropathy (IgAN). Also called Berger's disease, IgAN is a major cause of end-stage renal failure and the most common form of primary

glomerulonephritis. Renal damage in IgAN is caused by the deposition of IgA immune-complexes on mesangial cells and subsequent inflammatory responses. In IgAN patients, CD89 expression levels are reduced on myeloid lineage cells despite normal transcription, soluble CD89 concentrations are elevated in sera and CD89 is found on mesangial cells (Launay *et al.*, 1999; Monteiro, 2005). Additionally, transgenic CD89 mice develop IgAN-like pathogenesis (Launay *et al.*, 1999). A mechanism for IgAN pathogenesis has been proposed on the basis of the above mentioned observations. Abnormal IgA from IgAN patients interacts with CD89 on cells, resulting in the release of IgA/CD89 complexes into the circulation. These complexes then bind to mesangial cells through interaction with an IgA receptor and therefore induce inflammation (Launay *et al.*, 1999; Monteiro *et al.*, 2002; Monteiro, 2005). To date, the IgA receptor present on mesangial cells has not been characterized. It might be CD71 (the transferrin receptor) (Monteiro *et al.*, 2002), some uncharacterized IgA Fc receptor (Barratt *et al.*, 1999) or CD89 (Tsuge *et al.*, 2003). Although CD89 is not usually expressed on these cells, it has been argued that under yet to be established conditions its expression is induced and leads to production of the monocyte chemoattractant protein-1, which plays a role in the inflammatory process (Tsuge *et al.*, 2003). However, such a model of IgAN pathogenesis has been challenged. IgA/CD89 complexes have been described in serum of both IgAN patients and healthy individuals (van der Boog *et al.*, 2003), in discrepancy with reports of these complexes being unique to IgAN patients (Launay *et al.*, 1999). Injection of human CD89 into normal mice induces mesangial IgA deposits and mouse IgA is unable to bind human CD89 (van der Boog *et al.*, 2004). Thus, these findings are in apparent contradiction with

the results from the CD89 transgenic mouse models studies (Launay *et al.*, 2004).

Clearly, results obtained using mouse models of IgA/Fc receptor interactions must be interpreted with caution. Indeed, expression of IgA receptors is quite different between rodents and humans (Decot *et al.*, 2005).

Nonhuman primates are important animal models for biomedical research, including research that focuses on pathogenesis, vaccine development, transplantation and development of immunotherapeutics. IgA/CD89 interactions are likely to be important in several of these studies. For example, macaques are used as a model system for periodontal disease (Ebersole *et al.*, 2002) in which, as pointed out above, CD89 plays a role in pathogenesis. Previously, we have identified and partially characterized rhesus and cynomolgus macaque CD89 (Rogers *et al.*, 2004). Such characterization has been recognized as an important step in the development of animal models for the study of therapeutic antibodies and IgAN without the limitations that encumber mouse studies (Woof and Kerr, 2004).

Our further identification and characterization of CD89 in nonhuman primates shows these molecules to be functionally well conserved with human CD89 and provides new insights into the function of CD89 in humans as well. Nonhuman primate CD89 molecules share over 80% amino acid sequence identity to human CD89 and over 90% with each other (Table 3.1). Like macaque and human CD89 genes, baboon and sooty mangabey CD89 genes have 5 exons and produce multiple splice variants using different combinations of these exons. These splice variants may result in the production of functionally distinct CD89 isoforms (Figures 3.4-3.10). Similarly to human and macaque

CD89, baboon and sooty mangabey CD89 are expressed on blood leukocytes of the myeloid lineage only (Figures 3.13 and 3.14). There are different polymorphisms of human CD89, some of which are associated with increased disease risks (Kaneko *et al.*, 2003). Baboon and mangabey CD89 molecules are also polymorphic (Figures 3.10 and 3.11). Analysis of two baboon families shows that baboon CD89 gene polymorphisms follow a classical Mendelian inheritance (Figure 3.12), raising the possibility to identify polymorphisms associated with disease in these animals through analysis of pedigreed families.

Human IgA binds to human CD89 at the first extracellular domain (EC1) with interactions involving residues Y35, R52-N59, H85 and Y86 (Herr *et al.*, 2003). Half of these residues are substituted in Old World monkeys and in chimpanzees (Figure 3.2). Baboon and mangabey CD89 substitutions at these residues are nearly identical to those found in macaques. All of the Old World monkeys have the substitutions R52E, R53K, K54G, and Y86S. H85 is replaced in baboons and cynomolgus macaques with L85 and in sooty mangabeys and rhesus macaque with F85. These data suggest that CD89 from all four Old World species is likely to similarly interact with IgA. To assess nonhuman primate CD89 interactions with IgA, we chose to express rhesus macaque CD89, since this species is the most commonly used nonhuman primate for *in vivo* studies. We produced three HeLa cell clones expressing high levels of full length recombinant rhesus macaque CD89. Human IgA1 and IgA2 both bind to rhesus macaque CD89 indicating that CD89/IgA interactions are indeed conserved between humans and Old World monkeys (Figure 3.3).

Rhesus macaques possess a single *IGHA* gene that encodes the IgA heavy chain, which is highly polymorphic (Scinicariello and Attanasio, 2001; Scinicariello *et al.*, 2004). The functional properties of rhesus macaque IgA have not been previously studied. Therefore, our laboratory has generated a recombinant IgA molecule with affinity for the hapten NIP, containing rhesus macaque IgA CH1-CH3 domains, a mouse immunoglobulin light chain and a mouse variable heavy chain domain (Jayashankar, 2004). The deduced amino acid sequences of the recombinant rhesus macaque IgA CH1-CH3 domains are shown aligned with IgA CH1-CH3 sequences of humans and other primates in Figures 3.21-3.23. As depicted in these figures, human IgA residues responsible for forming interactions with human CD89 are located in the CH2 and CH3 domains. All but two of these residues are conserved in all nonhuman primates. In Old World monkeys there are substitutions (S46T and E48Q) in the CH3 domain. Therefore, interactions of different nonhuman primate IgA molecules with CD89 orthologues are predicted to be similar. Recombinant rhesus macaque IgA is capable of binding to recombinant rhesus macaque CD89 (Figure 3.17). Human CD89 is a low affinity receptor and does not bind to monomeric IgA with great efficiency. Affinity for IgA is increased when IgA binds to antigen and forms immune complexes (increased avidity). When the antigen of recombinant IgA was titrated out we observed that most of the detected IgA binding occurred regardless of the presence of antigen with a slight increase at low concentrations of antigen (Table 3.4). At the highest concentration of NIP (5) BSA-IgA binding decreased. Such a finding could be interpreted as follows: a) rhesus macaque CD89 has a relatively high affinity for monomeric IgA; b) addition of antigen at lower

CH1

	10	20	30	40	50
	:	:	:	:	:
Hu Igα1	ASPTSPKVFPLSLCSTQ	PDGNVVIACLVQGFFPQ	EPLSVTWSESGQGVTA		
Hu Igα2	ASPTSPKVFPLSLDSTP	QDGNVVVACLVQGFFPQ	EPLSVTWSESGQNVTA		
Mumu Igα	ASPTSPKVFPLNLCSTQ	SD-NVVVACLVQGFFPQ	EPLNVTWSKSGADVTV		
Soma Igα	ASPTSPKVFPLSLEGTQ	SD-NVVVACLVQGFFPQ	EPLNVTWNKSGAGVTV		
Mane Igα	ASPTSPKVFPLSLEGTQ	SD-NVVVACLVQGFFPQ	EPLNVTWSKSGAGVTV		
Paca Igα	ASPTSPKVFPLSLEGTQ	SD-NVVVACLVQGFFPQ	EPLSVTWNKSGAGVTV		

	60	70	80	90	100
	:	:	:	:	:
Hu Igα1	RNFPPSQDASGDLYTTSS	QLTLPATQCLAGKSVTCH	VKHYTNPSQDVTVPCP		
Hu Igα2	RNFPPSQDASGDLYTTSS	QLTLPATQCPDGKSVTCH	VKHYTNPSQDVTVPCP		
Mumu Igα	<u>I</u> NFPPSQDASGGLYTTSS	<u>Q</u> LTLPAA <u>Q</u> CP <u>A</u> SESVTCH	<u>V</u> EHYTNPSQDVA <u>V</u> PCV		
Soma Igα	<u>I</u> NFPPSQDASGGLYTTSS	<u>Q</u> LTLPAA <u>Q</u> CP <u>A</u> SESVTCH	<u>V</u> EHYTNPSQDVA <u>V</u> PCV		
Mane Igα	<u>I</u> NFPPRQDASGGLYTTSS	<u>Q</u> LTLPAA <u>Q</u> CP <u>A</u> SESVTCH	<u>V</u> EHYTNPRQDVA <u>V</u> PCR		
Paca Igα	<u>I</u> NFPPSQDASSGLYTTSS	<u>Q</u> LTLP <u>A</u> E <u>Q</u> CP <u>A</u> SESVTCH	<u>V</u> EHYTNPSQDVA <u>V</u> RCR		

Figure 3.21. Alignment of human (Hu), rhesus macaque (Mamu), sooty mangabey (Soma), pig-tailed macaque (Mane) and baboon (Paca) IgA CH1 deduced amino acid sequences. Residues that do not match human IgA1 residues are unlined. GenBank accession numbers are: human IgA1 J00220, human IgA2 J00221, Mamu IgA AY29614, and Soma IgA AY54480. Paca IgA and Mane IgA have not yet been assigned GenBank accession numbers. The Chinese rhesus macaque IgA sequence shown here is that of the gene used to produce recombinant IgA.

```

CH2
          10          20          30          40          50
          :          :          :          :          :
Hu Igα1  -VPSTPPTPSPSTPPTSPSPSCCHPRLSLHRPALEDLLLGSEANLTCTLTGL
Hu Igα2  -VP-----PPPPCCHPRLSLHRPALEDLLLGSEANLTCTLTGL
Mumu Igα -VPPITP---PC-----PSCCEPRLSLRRPALEDLLLGSEANLTCTLTGL
Soma Igα --PK----PNSC-----L-CDEPRLSLRRPALEDLLLGSEANLTCTLTGL
Mane Igα -VPP----PN-C----P-L-CDKPRLSLRRPALEDLLLGSEANLTCTLTGL
Paca Igα GIPP--P---P-----PS--CCEPRLSLHRPALEDLLLGSEANLTCTLTGL

          60          70          80          90          100
          :          :          :          :          :
Hu Igα1  RDASGVTFTWTPSSGKSAVQGPPERDLCGCYSVSSVLPGCAEPWNHGKTF
Hu Igα2  RDASGATFTWTPSSGKSAVQGPPERDLCGCYSVSSVLPGCAQPWNHGETF
Mumu Igα KDPSGATFTWTPSSGKNAVQQSPKRDPCGCYSVSSVLPGCAEPWNNRETF
Soma Igα RDPSGATFTWTPSSGKNAVQQSPERDPCGCYSVSSVLPGCAEPWNNRVTF
Mane Igα RDPSGATFAWTPSSGKNAVQQSPEHDPCGCYSVSSVLPGCAEPWNNRVTF
Paca Igα KDPSGATFTWTPSSGKNAVQQSPERDPCGCYSVSSVLPGCAEPWNNRETF

          110          120
          :          :
Hu Igα1  TCTAAYPESKTPLTATLSKS
Hu Igα2  TCTAAHPELKTPLTANITKS
Mumu Igα TCTANHPELETPLTATISKS
Soma Igα TCTANHPELETQLTATISKS
Mane Igα NCTANHPELKTPLTATISKS
Paca Igα TCTANHPELKTPLTATISKS

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Figure 3.22. Alignment of human (Hu), rhesus macaque (Mamu), sooty mangabey (Soma), pig-tailed macaque (Mane) and baboon (Paca) IgA CH2 deduced amino acid sequences, including the IgA hinge regions. Residues in human IgA1 as well as corresponding residues in other IgA molecules that make contact with human CD89 are shaded (Herr *et al.*, 2003). Residues that do not match human IgA1 residues are unlined. GenBank accession numbers are: human IgA1 J00220, human IgA2 J00221, Mamu IgA AY29614, and Soma IgA AY54480. Paca IgA and Mane IgA have not yet been assigned GenBank accession numbers. The Chinese rhesus macaque IgA sequence shown here is that of the gene used to produce recombinant IgA.

CH3

	10	20	30	40	50
	:	:	:	:	:
Hu Igα1	GNTFRPEVHLLPPPSEELALNELVTLTCLARGFSPKDVLRWLQGSQELP				
Hu Igα2	GNTFRPEVHLLPPPSEELALNELVTLTCLARGFSPKDVLRWLQGSQELP				
Mumu Igα	GNTFRPEVHLLPPPSEELALNELVTLTCLARGFSPEDVLRWLKGT <u>EQ</u> LP				
Soma Igα	GNTFRPEVHLLPPPSEELALNELVTLTCLARGFSPEDVLRWLKGT <u>EQ</u> LP				
Mane Igα	GNTFRPEVHLLPPPSEELALNELVTLTCLARGFSPEDVLRWLKGT <u>EQ</u> LP				
Paca Igα	GNTFRPEVHLLPPPSEELALNELVTLTCLARGFSPEDVLRWLKGT <u>EQ</u> LP				
	60	70	80	90	100
	:	:	:	:	:
Hu Igα1	REKYLTWASRQEPSQGTTTTFAVTSILRVAEDWKKGDTFSCMVGHEALPL				
Hu Igα2	REKYLTWASRQEPSQGTTTTFAVTSILRVAEDWKKGDTFSCMVGHEALPL				
Mumu Igα	RDKYLTWESRQEPSQGTTTTFAVTSILRVAEDWKKGDTFSCMVGHEALPL				
Soma Igα	RDKYLTWESRKEPSQGTTTTFAVTSILRVAEDWKKGDTFSCMVGHEALPL				
Mane Igα	RDKYLTWESRKEPSQGTTTTFAVTSILRVAEDWKKGDTFSCMVGHEALPL				
Paca Igα	RDKYLTWESRKEPNQGTTTTFAVTSILRVAEDWKKGDTFSCMVGHEALPL				
	110	120	130		
	:	:	:		
Hu Igα1	AFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY				
Hu Igα2	AFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY				
Mumu Igα	AFTQKTIDRLAGKPTHVNVSV				
Soma Igα	AFTQKTIDRLAGKPTHVNVSV				
Mane Igα	AFTQKTIDRLAGKPTHVNVSV				
Paca Igα	AFTQKTIDRLAGKPTHVNVSV				

Figure 3.23. Alignment of human (Hu), rhesus macaque (Mamu), sooty mangabey (Soma) and pig-tailed macaque (Mane) and baboon (Paca) IgA CH3 deduced amino acid sequences. Residues in human IgA1 as well as corresponding residues in other IgA molecules that form the surface interface with human CD89 are shaded (Herr *et al.*, 2003). Residues that do not match human IgA1 residues are unlined. GenBank accession numbers are: human IgA1 J00220, human IgA2 J00221, Mamu IgA AY29614, and Soma IgA AY54480. Paca IgA and Mane IgA have not yet been assigned GenBank accession numbers. The Chinese rhesus macaque IgA sequence shown here is that of the gene used to produce recombinant IgA.

levels resulted in IgA-immune complexes that bound with slightly higher affinity, but at higher concentration with excess antigen; c) IgA antigen binding sites were saturated and could not form larger immune complexes.

Differential glycosylation is characteristic of CD89 on different cells types and during certain types of pathogenesis (Monteiro *et al.*, 1992). Human CD89 can have N-glycans at four of the six N-glycosylation motifs (N44, N58, N120 and N156) (Herr, 2003). The other two sites are N165 and N177. All six of these N-glycosylation motifs are conserved in nonhuman primates. Additionally, macaque and baboon CD89 molecules have a seventh site at N4 that is absent in humans, chimpanzees and sooty mangabeys (Figure 3.2). Notably, N58 is found at the CD89/IgA interaction interface. Blocking of N-glycosylation greatly reduced the expression of rhesus macaque CD89 from HeLa cells and led to a greater decrease in IgA binding, indicating that N-glycans are important for both CD89 expression and interactions with IgA (Figure 3.18).

Alternative splice variants of human CD89 were identified over a decade ago. At least two isoforms are known to be produced (Monteriero and van de Winkel, 2003). The expression of these two isoforms is modulated by inflammatory cytokines (Togo *et al.*, 2003). However, neither the expression nor the function of the majority of these splice variants has been determined. Transfection of the genes for two alternative splice variants of rhesus macaque CD89 (CD89 Δ EC1 and CD89.9) into HeLa cells results in only very low expression on the cell surface (Figure 3.19).

The CD89 Δ EC1 splice variant has not been described in humans and would not be expected to be able to bind to IgA, since the domain responsible for IgA binding

(EC1) is deleted. The remaining domain (EC2) may play some as yet unknown functional roles. CD89 is most closely related to killer-inhibitory/activatory (KIR/KAR)-related immunoreceptors, Ig-like transcripts, as well as the leukocyte and monocytes/macrophage Ig-like receptors (Monteiro and van de Winkel, 2003). These molecules typically share about 35% identity with CD89 (Monteiro and van de Winkel, 2003). We have identified a putative pig KIR gene located upstream of the pig CD89 gene in a contig encoding an immunoglobulin-like domain. This domain is up to 42% identical to the EC2 domain of CD89 from different species. The homology of KIR/KAR molecules with CD89 suggests that they have some functions in common with CD89 EC2.

CD89.9 is a splice variant also found in humans and has a deletion of the exon encoding the transmembrane and cytoplasmic domains. This deletion results in the addition of an alternative tail from the translation of several codons found at the 5' end of the EC2-TM/C introns (Dijk *et al.*, 2006). Recombinant CD89.9 is mostly found in extracellular secretions and has been speculated to possibly contribute to CD89 deposits found on mesangial cells in IgAN (Dijk *et al.*, 1996; Monteiro *et al.*, 2002). Although CD89.9 can bind to IgA, it has not been determined yet whether or not it is produced *in vivo* and what its functional role maybe in health individuals. Here, we observed that media from cells transfected with a rhesus macaque CD89.9 expression vector could enhance CD89 cell surface staining on cells already expressing CD89 only in the presence of IgA. In addition, an apparent inhibition of IgA binding was observed. On the basis of these observations, it is reasonable to propose that CD89.9 may be produced *in*

vivo and may function under normal physiological conditions to modulate IgA activation of CD89 by blocking IgA binding. A single IgA molecule can bind to two CD89 molecules. Therefore, CD89.9 may act to prevent the initial binding of IgA to cells as well as bind to IgA already bound to a single CD89 molecule to block cross-linking and signal transduction through interaction with another membrane bound CD89 molecule. It would be of interest to know if this splice variant is expressed in species other than primates. As of yet similar splice variants have not been described in other species. The putative pig CD89 gene that we have identified has a EC1-TM/C intron 5' sequence similar to that of primates. CD89 sequences from pig and nonhuman primates are conserved (data not shown). In addition, a polyA tail motif (AATAAA) is present in the pig intron downstream of the stop codon, further supporting the presence of a similar splice variant in pigs.

Although the functions of IgA and CD89 are incompletely understood, it is clear that these molecules play roles in both protective immunity and autoimmunity. The presence of CD89 with similar expression and function profiles in four nonhuman primates strongly supports the development of animal models to study IgA and CD89 functions as well as to evaluate therapeutics.

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CHAPTER 4

Molecular Characterization of IgD in Mammals: Immunoglobulin Heavy Constant Delta (*IGHD*) Genes in Dogs, Chimpanzees and Four Old World Monkey Species

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Summary

Antibodies are adaptor molecules that neutralize pathogens and link humoral and cellular defense mechanisms. IgD, one of the five antibody classes present in mammals, is expressed as an antigen receptor on naïve B cells. The functional role that IgD plays in the immune response is still poorly understood, but the recent characterization of immunoglobulin heavy constant delta genes (*IGHD*) in a variety of species challenges the view that IgD is of minor importance and is not present in many animals. On the basis of serological studies, IgD appears to be expressed in the majority of mammalian species examined. To confirm, at the molecular level, that IgD is present in different species, we cloned and sequenced *IGHD* cDNA from dogs and five nonhuman primate species (chimpanzee, rhesus macaque, cynomolgus macaque, baboon and sooty mangabey). Our results show that in all six species, IgD heavy chains possess three immunoglobulin domains and a long hinge region encoded by two exons. Only the hinge region of

nonhuman primates is similar to the human hinge region, with conservation of O-glycosylation sites and multiple charged residues at opposing ends. The preservation of IgD in primates, dogs and previously characterized species suggests an important functional role for IgD, possibly involving binding to a receptor. The high degree of similarity existing between the structural features of human and nonhuman primate IgD suggests that nonhuman primates are suitable for *in vivo* studies designed to define the role that IgD plays in the immune response.

Introduction

Immunoglobulin D (IgD) is the least understood of the five antibody classes found in mammals, both from a functional and evolutionary perspective. On the surface of naïve B cells, IgD functions as an antigen receptor in apparent redundancy with IgM. Indeed, IgM heavy chain gene (*IGHM*) knockout mice appear to be healthy, with only a slight reduction of their B cell compartment (Lutz *et al.*, 1998). IgD is also present in a secreted form, with sera concentrations (40µg/ml in adults) considerably less than those of IgG, IgA and IgM (Preud'homme *et al.*, 2000). Attempts to identify the function of the secreted form have languished in part due to the difficulty of obtaining purified IgD (Preud'homme *et al.*, 2000). Initially described in 1965 as a human myeloma protein (Row and Fahey, 1965), IgD was subsequently characterized in humans, mice and rats at both the protein and genetic levels (Rowe and Fahey, 1965; Mushinki *et al.*, 1980; Putnam *et al.*, 1981; White *et al.*, 1981; Sire *et al.*, 1982; Zhao and Hammarström, 2003). The presence of IgD in nonhuman primates was firmly established soon after that of

human IgD (Martin *et al.*, 1976). Putative IgD molecules were also identified in other species including chicken, rabbit, dog and tortoise (Fiebig and Ambrosius, 1976; Eskinazi *et al.*, 1977; Sire *et al.*, 1977; Wilder *et al.*, 1979; Chen *et al.*, 1982; Yang *et al.*, 1995). Additional studies failed to identify IgD either at the protein or the genetic level in other species (swine, cows, sheep, duck and African clawed frog) as well as in the same species (chicken and rabbit) (Knight and Tunyaplin, 1995; Butler *et al.*, 1996; Naessens, 1996; Zhao *et al.*, 2000; Lundqvist *et al.*, 2001). The finding that mouse and human IgD are structurally different argued against a conserved IgD function (White *et al.*, 1985). These combined observations contributed to the speculation that IgD evolved recently, was repeatedly deleted in different species, and lacks a major function. However, results from recent studies indicate that IgD plays an important role in the immune system (Preud'homme *et al.*, 2000). IgD expression is differentially regulated from that of IgM (Loder *et al.*, 1999), and antigen binding properties differ for IgM and IgD due to differences in their hinge regions (Løset *et al.*, 2004). Antigen cross-linking of IgD on B cells leads to a stronger and more prolonged signal than that of IgM (Kim and Reth, 1995), and in IgD deficient mice affinity maturation is slower than that of normal mice (Roes and Rajewsky, 1993).

Analysis of newly described immunoglobulin heavy constant delta genes (*IGHD*) has greatly altered and expanded the understanding of IgD biology and evolution. Until the late 1990's, only human and mouse DNA sequences were available in addition to a partial rat sequence. Even with this paucity of genetic information, it is apparent that *IGHD* properties are quite divergent between species. The human *IGHD* consists of eight

exons: one encoding the first immunoglobulin domain (CH1), two encoding 58 amino acids of an extended hinge region (H1 and H2), two encoding the second and third immunoglobulin domains (CH2 and CH3), one encoding a hydrophilic secretory tail (CH-S), and two encoding the membrane tail (M1 and M2) (White *et al.*, 1985). By contrast mouse and rat *IGHD* have only 6 exons (CH1, a single hinge exon, CH3, CH-S, M1 and M2) (Mushinski *et al.*, 1980; Sire *et al.*, 1982; Zhao and Hammarström, 2003). The human IgD hinge region is characterized by a highly O-glycosylated N-terminal end encoded by H1 and a highly charged C-terminal end encoded by H2. The rodent IgD hinge region is shorter and appears to be structurally unrelated to the human hinge (Sire *et al.*, 1982). In 1997, Wilson *et al.* described an *IGHD* in channel catfish, which contains seven tandem immunoglobulin exons and lacks any hinge exon. *IGHD* has since been identified in Atlantic cod, Japanese flounder, carp, Atlantic salmon, Atlantic halibut, rainbow trout, fugu and zebra fish. These genes also encode IgD heavy chains without hinge regions and consist of various numbers of tandem immunoglobulin domain encoding exons, which for some species are repeated in clusters (Hordvik *et al.*, 1999; Stenvik and Jørgensen, 2000; Bengtén *et al.*, 2002; Hordvik, 2002; Hirono *et al.*, 2003; Saha *et al.*, 2004; Srisapoome *et al.*, 2004; Danilova *et al.*, 2005; Hansen *et al.*, 2005; Savan *et al.*, 2005). The fish IgD heavy chains are characterized by the fusion of their N-terminal end with the CH1 of IgM, which results in unique chimeric molecules. Recently, *IGHD* of cow, sheep, pig and horse have been sequenced. Their exon configuration is similar to that found in humans (Zhao *et al.*, 2002; Zhao *et al.*, 2003; Wagner *et al.*, 2004). With the exception of the pig IgD, which has the H2 exon spliced out (Zhao *et al.*,

2003), in ungulates the IgD hinge regions are all encoded by two exons. However, their hinge regions are dissimilar to those of humans and rodents in sequence. Interestingly, pig *IGHD* transcripts can have the IgM CH1 fused to their 5' end as described in fish (Zhao *et al.*, 2003). Together, these data demonstrate that *IGHD* has an ancient origin, is distributed widely across vertebrate taxa, and is structurally diverse particularly within the hinge region.

Despite the early recognition of the presence of IgD both on B-cells and as a secreted protein in nonhuman primates (Voormolen-Kalova *et al.*, 1974a; Voormolen-Kalova *et al.*, 1974b; Martin *et al.*, 1976; Finkelman and Scher, 1979; Black *et al.*, 1993), IgD has not been studied in these animals at the genetic level. In pioneering studies of IgD function, injection of anti-IgD antiserum into rhesus macaques was shown to enhance antibody responses to antigen in an adjuvant-like manner and lead to hypergammaglobulinemia, indicating a role for IgD in regulation of humoral responses (Pernis, 1975; Martin and Leslie, 1979). Because of their similarities to humans, nonhuman primates are commonly used as models to understand pathogenesis for a variety of human diseases and to develop therapeutic and preventive approaches (Kennedy *et al.*, 1997; Carlsson *et al.*, 2004). Although IgD in nonhuman primates appears well conserved with human IgD based on serology (Black *et al.*, 1993), more detailed studies are required to determine the extent to which this is so, particularly in light of the divergence of IgD seen in other species. A dog immunoglobulin with IgD-like properties, including B cell surface expression and lack of cross-reactivity with antibodies against the other dog isotypes, has been identified (Yang *et al.*, 1995). As

pointed out by Naessens (1996), conclusive evidence that this immunoglobulin is indeed canine IgD, such as cross-reactivity with known anti-IgD antibodies, is still lacking. Hence, the presence of IgD in dogs remains to be established, particularly since it has previously been proposed that IgD may have been deleted from many mammals (Naessens, 1996; Stenvik and Jørgensen, 2000).

The human IgD hinge region has structural features in common with the hinge region of IgA, including a repetitive sequence. It has been suggested that this repetition is possibly responsible for the genetic instability and diversification of the IgA hinge region (Flanagan *et al.*, 1984). If this is correct, the hinge regions of IgD from species closely related to humans might be expected to be highly divergent and polymorphic. On the other hand, the O-glycans of the human hinge are responsible for forming interactions with an IgD receptor expressed on human CD4⁺ and CD8⁺ T cells (Tamma and Coico, 1992; Rudd *et al.*, 2001). If the human IgD receptor has orthologues in nonhuman primates and it is important for survival, then the hinge region of nonhuman primates may be well conserved with that of human IgD. Therefore, we have sequenced *IGHD* from chimpanzee, rhesus macaque, cynomolgus macaque, baboon and sooty mangabey. We have also sequenced dog *IGHD*, thus confirming the presence of IgD in carnivora, and expanding the growing body of evidence that IgD is present in most mammals and likely to be functionally important.

Materials and methods

Blood samples and RNA extraction

Total RNA was extracted from heparinized whole blood of two rhesus macaques (*Macaca mulatta*), three cynomolgus macaques (*Macaca fascicularis*), two baboons (*Papio hamadryas anubis*) and one sooty mangabey (*Cercocebus torquatus*), whereas total RNA was extracted from isolated PBMC of two chimpanzees (*Pan troglodytes*) and one dog (*Canis familiaris*) using the QIAamp RNA Blood Mini Kit (Qiagen Inc., Valencia, CA). PBMC were isolated from whole blood by Histopaque®-1077 (Sigma-Aldrich Corp., St. Louis, MO) centrifugation. All animals used were healthy. Macaques and baboons samples were from animals housed at the Southwest National Primate Research Center San Antonio, TX. The sooty mangabey and the chimpanzees, Tika and Manuel, were housed at the Yerkes National Primate Research Center, Emory University, Atlanta, GA. Whole dog blood was purchased from Harlan Bioproducts for Science, Inc., Madison, WI.

Amplification, cloning and sequencing of immunoglobulin D heavy chain cDNAs

RNA was reverse transcribed into cDNA using oligo d(T)17 primers, followed by primer extension with AMV reverse transcriptase (Roche Diagnostic Corp., Indianapolis, IN). PCR amplification of cDNA was performed with Expand High Fidelity polymerase (Roche Diagnostic Corp.). Primers for amplification of primate *IGHD* were designed on the basis of the human sequence assuming conserved homology between primates. The

forward primer IgD7 (5'-CGGATGTGTTCCCCATCATATCAG-3') is located in the 5' end of the human CH1 exon (16-39nt). Two reverse primers were used with IgD7, IgD3 (5'-ACCCAGAAGTGTTCACCTCACG-3') located in the center of the CH3 exon (135-156nt) and IgD13 (5'-AGCTGACTTCTAGGCTCCGGCT-3') located at the 3' end of CH3 exon (303-324nt). Canine primers were designed from DNA sequences of a contig (GenBank accession number NW_140211) predicted to encode an IgD heavy chain-like protein. The canine forward primer K9IGHDF1 (5'- ATCGTCACTTCTGCTCCCCTTG -3') is located in the canine IgD CH1 exon (12-33nt). The canine reverse primer K9IGHDB6 (5'- AGCAAAAAGGCAAGGGGCTG -3') is located in a region upstream of a polyadenylation signal and downstream of the M2 exon. After initial denaturation at 95°C for 10 min, cDNAs was amplified for 40 cycles, with each cycle consisting of 94°C for 1 min, 56°C for 1 min and 72° for 1 min 30 sec. A final step at 72°C for 10 min was used to ensure complete extension. Primers IgD7 and IgD13 yield a human product of 1110bp and primers IgD7 and IgD3 yield one of 942bp. All reactions were performed in at least two independent reverse transcriptions PCR to verify product sequences. At least 10 clones were sequenced for each animal of the primate species and six clones were sequenced for dog. An additional primer set was used to amplify the baboon IgD hinge region exons and the immediate surround nucleotides (BGDF 5'- AGTACAAATGCACCGTCAAGCAT-3' and BGDR 5'- CGAAGCAGGTGAAGGTGACTTTG-3').

Cloning of the amplified gene sequences

For cloning, 100µl of a reverse transcription PCR was run on a 1% agarose gel. The specific band of interest was excised from the gel and purified using a QIAquick® Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). The cDNA was ligated into TopoTA vector and transformed into Top10 *Esheria coli* (Invitrogen Corp., Carlsbad, CA). Plasmid DNA was purified using a FastPlasmid® Mini kit (Eppendorf, Inc., Hamburg, Germany) and screened on a 1% agarose gel after digestion with *EcoRI* to confirm the correct size of the DNA fragments. All DNA sequences were determined using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3100 Genetic Analyzer (PerkinElmer, Inc., Wellesley, MA). The forward and reverse M13 primers were used for the sequencing. For the canine DNA fragments, an additional primer, K9IGHDB2 (5'- TGATCCAGGTGAGGAGGATGTCAG -3'), located in the CH3 exon, was used.

Analysis of DNA sequences

Overlapping regions were identified and sequences were edited using the MacVector software program (Accelrys Inc., San Diego, CA). Sequences were aligned with each other and other known *IGHD* using the CLUSTAL function of the MEGALIGN part of the LASERGENE software package (DNASTAR Inc., Madison, WI). ImMunoGeneTics (IMGT) standardized nomenclature and numbering has been used to show and discuss data based on human reference sequences (Lefranc and Lefranc, 2001). The GenBank accession numbers for the *IGHD* sequences of the various species

used for analysis which were previously available are human K02875-K02883, horse AY631942, cow AF411240, pig AF411239 and AY228508, sheep AF411238, rat AY148494 and AY148495, and mouse V00786-V00788 and J00450,. The GenBank accession numbers for the *IGHD* sequences described in this study are chimpanzee DQ297173-DQ297174, baboon DQ297175-DQ297176, cynomolgus macaque DQ297177-DQ297178, rhesus macaque DQ297179-DQ297181, sooty mangabey DQ297182-DQ297184 and dog DQ297185.

Results

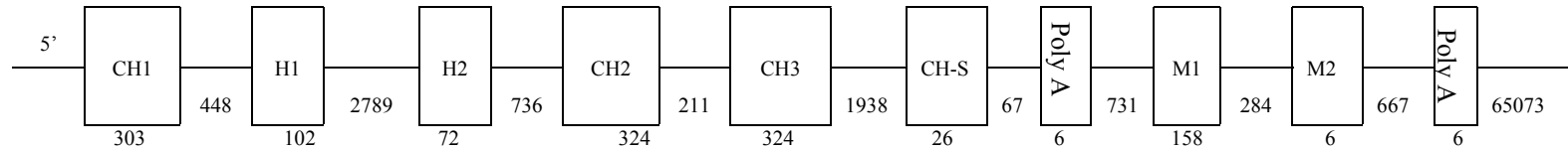
Chimpanzee IGHD

We cloned and sequenced *IGHD* using total RNA of PBMC from two different chimpanzees. Tika and Manuel's *IGHD* were sequenced using the primers IgD7 and IgD3, which amplify a 942bp product with a sequence encoding amino acids from the CH1 position 7 to the CH3 position 52. Tika's *IGHD* was also amplified using the primer pair IgD7 and IgD13 allowing for sequencing through the 3' terminus of the CH3 and yielding a 1110bp product. Attempts to sequence Manuel's *IGHD* with this latter primer pair were unsuccessful. The region amplified for both animals had identical sequence except for a single G/C difference in the CH1 codon 14 at position 3, which is silent. Through screening of GenBank for matches with the chimpanzee mRNA sequences *IGHD*, the genomic sequence was identified on a contig from the chimpanzee chromosome 14 (NW_115908). The nucleotide sequence of the contig has 99.4% identity to that of the cDNA, with a G in the third position of CH1 codon 14. In the contig

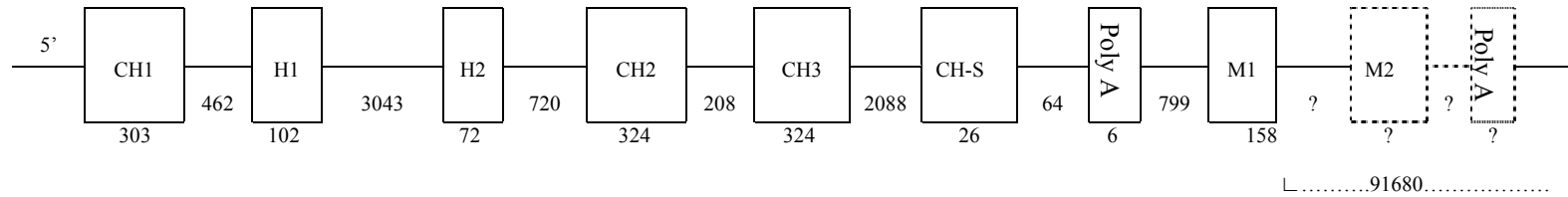
sequence three_nucleotide differences from the cDNA sequences are present: an insertion of C at CH2 codon 16 between positions 2 and 3, which results in a reading frame shift; a silent A to C substitution of CH2 codon 24 position 3; and a missense A to C substitution of CH3 codon 106 at position 3 resulting in a coding change from glutamate to aspartate. All exons had the anticipated boundaries and were of equivalent sizes to the human counterparts. Besides the CH1-3 identified by reverse transcription PCR, we identified an exon for the secretory tail and the first exon for the transmembrane tail from the contig (Figures 4.1 and 4.2).

The chimpanzee *IGHD* deduced amino acid sequence, which is 98.1% identical to that of human *IGHD*, is shown aligned with *IGHD* of other species in Figure 4.2. All cysteines responsible for inter- and intra- chain disulfide bonds found in human *IGHD* are conserved in the chimpanzee (CH1 positions 15, 28 and 84; CH2 positions 2, 31 and 90; CH3 positions 27 and 88). Similarly, the three N-glycosylation motifs (CH2 asparagine 66 and CH3 asparagines 49 and 100) are conserved between humans and chimpanzees. The human IgD N-terminal portion of the hinge is highly O-glycosylated (Putnam *et al.*, 1981). Identified sites of O-glycosylation in human IgD hinge include H1 encoded residues S8, S9, T12, T25, T26, T30 and T31. All of these O-glycosylation sites are present in chimpanzee IgD with the exception of T25, which is replaced by an arginine in chimpanzee.

Human Chromosome 14q32.33 (GenBank accession NC_000014)



Chimpanzee Chromosome 14



Dog contig NW_140211

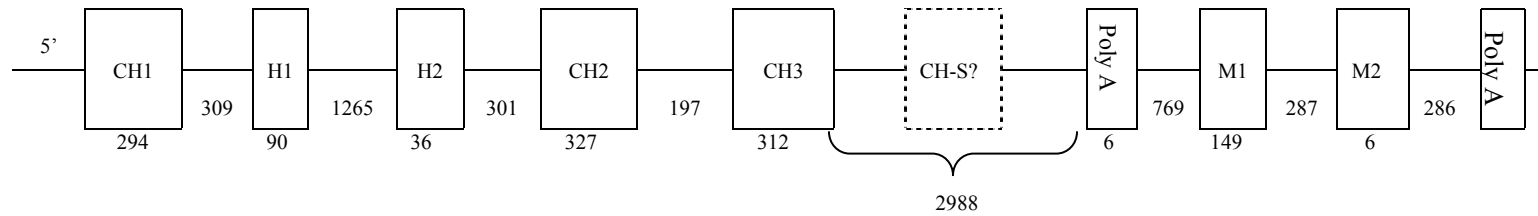


Figure 4.1. Chain diagram of human, chimpanzee and dog *IGHD* genes. Numbers indicate nucleotides in exons or introns.

Domains which have not been identified are indicated by dashed lines.

Figure 4.2. (Following pages) Alignment of IgD heavy chain deduced amino acid sequences for each domain: CH1 (A), hinge H1 (B) and H2 (C), CH2 (D), CH3 (E) and transmembrane (F). Mouse and rat hinge is encoded by a single exon. Pig H2 is spliced out of the mature mRNA and its deduced sequence is denoted in italics case to reflect this (Zhao *et al.*, 2003). B) Glycines in the middle of the hinge, potentially contributing to flexibility, are underlined. O-glycan sites in human H1 and the corresponding conserved residues in nonhuman primates are bolded and italicized. A, C, D, and E) N-glycosylation motifs (NXS or NXT where X is not proline) are bolded and italicized. Cysteines that form disulfide bonds within immunoglobulin domains and between immunoglobulin chains are bolded and underlined. F) Amino acid of the conserved antigen receptor transmembrane motif (CART) are underlined and bolded. Mangabey.1 and mangabey.2 in D are two sequence variants. Numbering is based on IMGT numbering for human IgD heavy chain and disregards insertions and deletions found in other species. GenBank accession numbers are given in the methods sections.

A

IgD CH1	1	20	40
Human	APTKAPDVFPIISG <u>C</u> RHPKDNSPVVLA <u>C</u> LITGYHPTSVTV		
Chimpanzee	APTKAPDVFPIISG <u>C</u> RHPKDNSPVVLA <u>C</u> LITGYHPTPVTV		
Baboon	DVFPIISV <u>C</u> ELPKDNSPVVLA <u>C</u> LITGYNPKSVTV		
Mangabey	DVFPIISAC <u>C</u> QLPKDNSPVVLA <u>C</u> LITGYNPKSVTV		
Rhesus	DVFPIISAC <u>C</u> QLPKDNSPVVLA <u>C</u> LITGYNPKSVTV		
Cynomolgus	DVFPIISAC <u>C</u> QLPKDNSPVVLA <u>C</u> LITGYNPKSVTV		
Dog	ASRESSLLLPLVSG <u>C</u> KVPKNGEDITLA <u>C</u> LAKGPF LDSVRV		
Horse	SLEDTAVIPLFSE <u>C</u> KAPKEDDVVSLA <u>C</u> LVKGYFPEPVQV		
Cow	EGESHLRVFPLVS <u>C</u> VSSPSDESTVALG <u>C</u> LARDFVPNSVSF		
Sheep	ESESHPKVFPLVS <u>C</u> VSSPSDENTVALG <u>C</u> LARDFMPNSVSF		
Pig	ESQSAPNLYPLVS <u>C</u> VSPPSDESLVALG <u>C</u> LARDFLPSSVTF		
Mouse	NFT ICLAGDKKEPDMF-LLSE <u>C</u> KAPEENEKINLG <u>C</u> LVIGSQP--LKI		
Rat	APEKEPDLF-LSSE <u>C</u> KAPNQNEHV <u>NVSC</u> MAIGVQP--LTL		
	41	60	80
Human	TWYMG-----QSQPQRTFPEIQRRDSYYMTSSQLS---TPLQQWRQG		
Chimpanzee	TWYMG-----QSQPQRTFPEIQRRDSYYMTSSQLS---TPLQQWRQG		
Baboon	TWHLGT-----QIQNQIMFPETER-EGSYTTSSQLAP-TPLLSQQHQG		
Mangabey	TWHLGT-----QIQNQIMFPETER-EGSYMTSSQLAP-TPPLSQQHQG		
Rhesus	TWHLGT-----QIQNQIMFPETER-EGSYTTSSQLAP-TPPLSQQHQG		
Cynomolgus	TWHLGT-----QIQN Q TMFPETER-EGSYTTSSQLAP-TSPLSQQHQG		
Dog	TTGPES-----QAQMEKTTLKMLK-IPDHTQVSLLS-----TPWKPG		
Horse	TWPEM-----QNQKPWTFPAMKK-GQEYIHVFSLT-----TWWKPG		
Cow	SWK FNST -VSSERFWTFPEVLR-DGLWSASSQVVLPSSSAFQGPDD		
Sheep	SWKL FNST -VSSERFWTFPEVLR-DGLWSASSQVALHSSSAFQG-TD		
Pig	SWNYK NSSK VSSQNIQDFPSVLR-GGKYLASSRVLLPSVSIQDPEA		
Mouse	SWEPPK-----SSIVEHVFPSEMR-NG NYT MVLQVT---VLASEL-NL		
Rat	AWEP-----TLPSTVFSGKNK-D-EYITILQVS---VPAP NL -SP		
	81	100	
Human	EYK <u>C</u> VVQHTASKSKKE-IFRWP		
Chimpanzee	EYK <u>C</u> VVQHTASKSKKE-IFRWP		
Baboon	EYK <u>C</u> TVKHTPSNTNQEKTFRWP		
Mangabey	EYK <u>C</u> TVKHIPSDTNKAKTFRWP		
Rhesus	EYK <u>C</u> TVKHTPSDTSKEKTFRWP		
Cynomolgus	EYK <u>C</u> TVKHTPSNTNNEKTFRWP		
Dog	LHY <u>C</u> EAIRKDNKEKLLKAIHWP		
Horse	SHS <u>C</u> TVHHKASSFRKKMTFQEP		
Cow	YLV <u>C</u> EVQHPKGGKTVGTVRVVPR		
Sheep	GYL <u>C</u> EVQHPKGGKTVGTVRVVPR		
Pig	FLV <u>C</u> EVQHPSGTSVSIISGP		
Mouse	NHTC TINKP---KRKEKPFKFP		
Rat	NLTC TIT N TS--KKKSKTFKLP		

Figure 4.2. A.

B

IgD Hinge H1

	1	20
Human		
Human	ESPKAQASSVPTAQPQAEGSLAKA	TTAPAT
Chimpanzee	ESPKAQASSVPTAQPQAEGSLAKA	TRAPAT
Baboon	ESPKAQYPSVPTVQPQAEGGLSKA	TRPPAT
Mangabey	ESPKAQYPSVPTVQPQAEGGLSKA	TRPPAT
Rhesus	ESPKAQYPSVPTVQPQAEGGLSKA	TRPPAT
Cynomolgus	ESPKVQYPSVPTVQPQAEGGLSKA	TRPPAT
Dog	ASWETAIS-LLTHAPSR---	PQDHTQAPSMARVS
Horse	ASWAPQ-----	RTSALPVTSKEPTPAPTTLRKS
Cow	ASTPT--P-T-TPLPSLISGSEGSNKAVSTQSSP	
Sheep	VSASTLTP-T-TLAPSLKSRSEGSSKAVTTQSSP	
Pig	ASRQLPAP-AGTPGPTL---	STVSTKALTTPRIPA
Mouse	-SWDSQSSKRVTPTLQAK	NHSTEATKAITTKKDIE
Rat	ETRNSQSSKKANPTPQAKNH	YIEATKPTATKNIVG

C

IgD Hinge H2

	1	20
Human		
Human	G	R
Chimpanzee	G	R
Baboon	G	R
Mangabey	G	R
Rhesus	G	R
Cynomolgus	G	R
Dog	V	P
Horse	E	P
Cow	A	L
Sheep	V	P
Pig	-	R

Figure 4.2. B and C.

D

IgD CH2

	1	20	40
Human	E <u>C</u> PSHTQPLGVYLLTPAVQDLWLRDKATFT <u>C</u> FVVGSDLKD		
Chimpanzee	D <u>C</u> PSHTQPLGVYLLPPAVQDLWLRDKATFT <u>C</u> FVVGSDLKD		
Baboon	E <u>C</u> PSHTQPLGVYLLPPALQDLWFRDKVTFT <u>C</u> FVVGSDLQD		
Mangabey.1	E <u>C</u> PSHTQPLGVYLLPPALQDLWFRDKVTFT <u>C</u> FVVGSDLQD		
Mangabey.2	E <u>C</u> PSHTQPLGVYLLPPALQDLWFRDKATFT <u>C</u> FVVGSDLQD		
Rhesus	E <u>C</u> PSHTQPLGVYLLPPALQDLWFQDKVTFT <u>C</u> FVVGSDLQD		
Cynomolgus	E <u>C</u> PSHTQPLGVYLLPPALQDLWFQDKAIFT <u>C</u> FVVGSDLQD		
Dog	E <u>C</u> WNHT HPPSLYMLRPPPLRGPWLQGEAAFT <u>C</u> LVVGDDLQK		
Horse	E <u>C</u> QSHTHPPSIYLLHPPLOGLWLKGEATFT <u>C</u> LVVGDDLKD		
Cow	E <u>C</u> QNHT QAPRVHLLPPTPQGLWLLDKAEFT <u>C</u> LATGEAPLD		
Sheep	E <u>C</u> QNHT QAPSVRLPPPPQGLWLLDKAEFT <u>C</u> LATGEALLD		
Pig	- <u>C</u> QNHT RPPSVYLLLPPLQGLWLRAEATFT <u>C</u> LAVGQDLQE		
	41	60	80
Human	AHLTWEVAGKVPTGGVEEGLLERHS NGS QSQHSRLTLPRS		
Chimpanzee	AHLSWEVAGKVPTGGVEEGLLERHS NGS QSQHSRLTLPRS		
Baboon	AHLSWEVAGKVPKGDMEEGPLEQHS NGS QSQHSRLALPRS		
Mangabey.1	AHLSWEVAGKVPKGGMEEGPLEQHS NGS QSQHSRLALPRS		
Mangabey.2	AHLSWEVAGKVPEGGMEEGPLEQHS NGS QSQHSRLALPRS		
Rhesus	AHLSWEVAGKVPKGGMEEGPLEQHS NGS QSQHSRLALPRS		
Cynomolgus	AHLSWEVAGKDPKGGMEKGPLEQHS NGS QSQHSRLALPRS		
Dog	AHLSWEVAGAPPSEAVEERPLQEHE NGS QSWSSRLVLPIS		
Horse	AHLSWELSERSNGMFVESGPLEKHT NGS QSRSSRLALPRS		
Cow	AHFSWEVNGQPHGGALEEG-PT RHI NSS WSQSSRLALPRS		
Sheep	AHFSWEVNGQPHGGAVEER-PT SHM NGS WSHSSRLALPRS		
Pig	ARLSWAVAEDPQGGRMEEG-PT EHT NGS WSLSRLALPRS		
	81	100	
Human	LWNAGTSVT <u>C</u> TLNHPSLPP-QRLMALREP		
Chimpanzee	LWNAGTSVT <u>C</u> TLNHPSLPP-QRLMALREP		
Baboon	LWNAGTSVT <u>C</u> TLNHPSLPS-QKLMALREP		
Mangabey.1	LWNAGTSVT <u>C</u> TLNHPSLPS-QKLMALREP		
Mangabey.2	LWNAGTSVT <u>C</u> TLNHPSLPS-QKLMALREP		
Rhesus	LWNAGTSVT <u>C</u> TLNHPSLPS-QKLMALREP		
Cynomolgus	LWNAGTSVT <u>C</u> TL NHS SLPS-QKLMALREP		
Dog	LWASG ANITC TL S LPSPMPQVVSAAAREH		
Horse	SWAMGTSVT <u>C</u> KLSPNLLSSMEVVGLKEH		
Cow	LWASGS NVTC TLSSPGLQSPVTLTAQREH		
Sheep	LWASGS NVTC TLSSPGLRSPVSLTAQREH		
Pig	SWAAGAPVT <u>C</u> RLSGPGLRSLVTAEAREH		

Figure 4.2. D.

E

IgD CH3	1	20	40
Human			
Human	AAQAPVKLSLNLLASSDP---	PEAASWLL	<u>CEVSGFSPPNILLM</u>
Chimpanzee	AAQAPVKLSLNLLASSDP---	PEAASWLL	<u>CEVSGFSPPNILLM</u>
Baboon	AAQAPVRLSLNLLASSDP---	PEAASWLL	<u>CEVSDFSPNILLM</u>
Mangabey	AAQAPVRLSLNLLASSDP---	PEAASWLL	<u>CEVSDFSPNILLM</u>
Rhesus	AAQAPVRLSLNLLASSDP---	PEAASWLL	<u>CEVSDFSPNILLM</u>
Cynomolgus	AAQAPVRLSLNLLASSDP---	PEAASWLL	<u>CEVSDFSPNILLM</u>
Dog	AARAPSSLNVHALTMP-----	RAASWFL	<u>CEVSGFSPPDILLT</u>
Horse	AASAPRSLTVHALTTPGL	NAS PGATSWLQ	<u>CKVSGFSPPEIVLT</u>
Cow	AASVPG NLTL RLRTVTAPG----	PFSPA	<u>WLLCEVSGFSPVDILLT</u>
Sheep	AASVPG NLTL RLRLTTPG----	PFSPA	<u>WLLCEVSGFSPVDILLT</u>
Pig	AALAPSNLAVRVLTA	PGPLAFTKAASWLL	<u>CEVSSFSPLDILLT</u>
Mouse	AMAPS NLTV NILTTSTH---	PEMSSWLL	<u>CEVSGFFPENIHLM</u>
Rat	AMAPSNLNVNILTTFTH---	HEMSSWLM	<u>CEVSGFYEDIHLW</u>
	41	60	80
Human			
Human	WLEDQREV	NTS GFAPARPPPQPRSTTF	WAWSVLRVPAPPS
Chimpanzee	WLEDQREV	NTS GFAPARPPPQPGSTTF	WAWSVLRVPAPPS
Baboon	WLEDQREV	NTS WFATTHPTPQPGSTM	FWAWSVLRVPGPTS
Mangabey	WLEDQREV	NTS WFATTHPTPQPGSTM	FWAWSVLRVPGPTS
Rhesus	WLENQREV	NTS WFATTHPTPQPGSTM	FWAWSVLRVPGPTS
Cynomolgus	WLEDQREV	NTS WFATTHPTPQPGSTM	FWAWSVLRVPGPTS
Dog	WIKDQIEVDPSWFATAPPMAQ	PGSGTFQ	TWSLLRVLAPQG
Horse	WLEGQREVDPSWFATARPTAQ	PG NTT FQ	TWSILLVPTIPG
Cow	WLEGQQEVVEPSQFATAHTTAQ	AGRASSHT	TWSVLRVSSPLD
Sheep	WLEGQQEVVEPSQFATAHTTAQ	SGHASFHT	TWSVLHVSSPLD
Pig	WLEGQQEVVDPSWFATARPAAQ	PGNPTFRT	TWSVLRVPASPG
Mouse	WLGVHSMKSTNFVTANPTAQ	PGG-TFQ	TWSVLRRLPVALS
Rat	WLSAQTKMDPINFVTAQ	PVRQSGD-KFQ	IWSVLRRLPVALS
	81	100	
Human			
Human	PQPATYTT	<u>CVVSHEDSR</u> TLL NAS RSLEVS	
Chimpanzee	PQPATYTT	<u>CVVSHEDSR</u> TLL NAS RSLEVS	
Baboon	PQPATYTT	<u>CVVSHEDSR</u> TLL NAS RSLEVS	
Mangabey	PQPATYTT	<u>CVVSHEDSR</u> TLL NAS RSLEVS	
Rhesus	PQPATYTT	<u>CVVSHEDSR</u> TLL NAS RSLEVS	
Cynomolgus	PQPATYTT	<u>CVVSHEDSR</u> TLL NAS RSLEVS	
Dog	PHPPTYTT	<u>CVVRHEASR</u> KLL NTS WSLDS	
Horse	PPTATYTT	<u>CVVGHEASR</u> QLL NTS WSLDTG	
Cow	HAGATYTT	<u>CVVSHEASR</u> TLL NGS CSLDT	
Sheep	HVGSTYTT	<u>CVVSHEASR</u> TLL NGS CSLDTG	
Pig	HQDATYTT	<u>CVVGHEASR</u> TLL NAS WRLDTG	
Mouse	SSLDTYTT	<u>CVVEHEASK</u> TKL NAS KSLAIS	
Rat	PSLDTYTT	<u>CVVEHEASQ</u> TKL NAS KSLEIS	

Figure 4.2. E

F

IgD TM	1	20	40
Human	YLAMTPLIPQSKDENSDDYTTFDDVGS	LWT	<u>TL</u> <u>ST</u> <u>F</u> <u>VAL</u> <u>F</u> <u>IL</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>I</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u> <u>V</u> <u>K</u>
Chimpanzee	DLAMTPLIPQSKDENSDDYTTFDDVGS	LWT	<u>TL</u> <u>ST</u> <u>F</u> <u>VAL</u> <u>F</u> <u>IL</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>I</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u> <u>V</u> <u>K</u>
Dog	GLTMTPPAPQSHDESSGDSMDLEDASGLWP---		<u>T</u> <u>F</u> <u>A</u> <u>A</u> <u>L</u> <u>F</u> <u>V</u> <u>L</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>L</u> <u>K</u> <u>V</u> <u>K</u>
Horse	GLAMTPE---SKDENSDDYADLDDAGSLWL---		<u>T</u> <u>F</u> <u>M</u> <u>A</u> <u>L</u> <u>F</u> <u>L</u> <u>I</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u>
Cow	GLATWPPW--SQDESSDDGTDVEDASPLWL---		<u>T</u> <u>F</u> <u>L</u> <u>A</u> <u>L</u> <u>F</u> <u>L</u> <u>V</u> <u>T</u> <u>V</u> <u>V</u> <u>Y</u> <u>G</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u> <u>V</u> <u>K</u>
Sheep	GLATWPPW--SQDESSDDSADAEDASPLWL---		<u>T</u> <u>F</u> <u>L</u> <u>A</u> <u>L</u> <u>F</u> <u>L</u> <u>A</u> <u>T</u> <u>V</u> <u>V</u> <u>Y</u> <u>S</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u> <u>V</u> <u>K</u>
Pig	GLATLTPG--SQDEGSDDYVDLEDAGRLWL---		<u>T</u> <u>F</u> <u>T</u> <u>V</u> <u>L</u> <u>F</u> <u>L</u> <u>V</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>L</u> <u>K</u> <u>V</u> <u>K</u>
Mouse	GIVNTIQHSCIMDEQSDSYMDLEEENGLWPT	<u>M</u> <u>C</u> <u>T</u> <u>F</u> <u>V</u> <u>A</u> <u>L</u> <u>F</u> <u>L</u> <u>L</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u> <u>V</u> <u>K</u>	
Rat	GMVDTIPNSCIRDEQTDSYVDLEEENGLWPT	<u>L</u> <u>L</u> <u>C</u> <u>T</u> <u>F</u> <u>V</u> <u>A</u> <u>L</u> <u>F</u> <u>L</u> <u>L</u> <u>TLL</u> <u>YS</u> <u>G</u> <u>F</u> <u>VT</u> <u>F</u> <u>I</u> <u>K</u> <u>V</u> <u>K</u>	

Figure 4.2. F.

IGHD mRNA transcripts in Cercopithecoidea

With the same strategy used to clone and sequence chimpanzee *IGHD*, we identified *IGHD* transcripts for four Old World monkey species commonly used in biological research. *IGHD* were sequenced for two rhesus macaques, three cynomolgus macaques, two baboons and one sooty mangabey. Amplification of monkey *IGHD* cDNA with the primer pair IgD7 and IgD13 resulted in an 1107bp product for all four species. Because *IGHA* in macaques and baboons is highly polymorphic (Scinicariello and Attanasio, 2001; Scinicariello *et al.*, 2004), multiple animals were used for some of the species to allow for identification of potentially high levels of *IGHD* polymorphisms in these species. A single *IGHD* sequence was present for each species, except sooty mangabey, which had two sequences in one animal that varied at five nucleotides resulting in two amino acid substitutions. As discussed below, polymorphisms of *IGHD* appear to be less extensive than those of *IGHA* in nonhuman primates.

The deduced amino acid sequences for each species are shown in Figure 4.2. The percent identities of the Old World monkey sequences with those of the human and chimpanzee (in parentheses if different) sequences are 72% baboon, 77% (78%) sooty mangabey, 77% rhesus macaque and 76% cynomolgus macaque. The percent identities between Old World monkeys are higher: 96% baboon-sooty mangabey, 97% baboon-rhesus macaque, 94% baboon-cynomolgus macaque, 98% sooty mangabey-rhesus macaque, 96% sooty mangabey-cynomolgus macaque and 97% rhesus macaque-cynomolgus macaque (Table 4.1). The transcripts encoded all expected domains: CH1, hinge, CH2 and CH3. In the Old World monkeys, CH1 is two amino acids longer than

Table 4.1. Range of percent identities for deduced amino acid sequences between the IgD heavy chain domains of different mammalian phyla. Groups used are based on representative species for which *IGHD* has been identified and include: hominoids (human and chimpanzee); cercopithecoidea (baboon, mangabey, rhesus macaque and cynomolgus macaque); primates (combined cercopithecoidea and hominoids); Laurasiatheria (dog, horse, pig, sheep and cow); rodents (mouse and rat). * For the described species of rodents, IgD does not encode a CH2 domain.

	CH1	Hinge	CH2	CH3	CH1-3
Human-Chimpanzee	98.9	96.6	97.2	99.1	98.1
Hominoids-Cercopithecoidea	57.7-58.8	61.8-65.5	84.3-89.8	88.9-90.7	71.5-77.7
Within Cercopithecoidea	91.8-95.9	96.4-100	93.5-99.1	99.1-100	93.8-98.4
Primates-Laurasiatheria	21.6-32.0	13.2-19.6	48.6-55.6	51.4-60.2	27.3-42.4
Primates-Rodents	15.5-18.7	23.5-31.4	N/A *	47.2-51.9	12.9-32.3

the hominoid CH1 (excluding any differences in the first six amino acid which are encoded by nucleotides prior to where our forward PCR primer annealed), while the Old World monkey hinge is three amino acids shorter than the hominoid IgD hinge. These differences result in Old World monkey IgD heavy chains being one amino acid shorter in their described CH1-CH3 portion than is found in chimpanzee and human IgD heavy chain. Comparing hominoids to Old World monkeys, CH1 of Old World monkeys lacks arginine 60, has an insertion of proline and threonine between S71 and T72, and an insertion of lysine between E96 and I97. Within the carboxy-terminal half of the hinge region, three amino acids present in human and chimpanzee *IGHD* are absent in the Old World monkeys: G3, G4 and R19. All N-glycosylation motifs and cysteines involving inter- and intra-chain disulfide bonds in humans are conserved in Old World monkey IgD. *Cynomolgus* macaques have additional potential N-glycosylation sites at CH1 N50 and CH2 N93. These positions are not N-glycosylated in any of the other known mammalian IgD. CH1 N50 is also present in pig and the other Old World primates and CH2 N93 is present in all the primates examined. However, in these species, neither is followed by the N-glycosylation consensus sequence. Human O-glycosylation sites in the first half of the hinge are partially conserved in the Old World monkeys; H1 encoded residues S9, T12, T25 and T30 are conserved, but S8, T26, T31 are proline, arginine and asparagine respectively in Old World monkeys.

Unexpectedly, in addition to the 1107bp inserts from the RT-PCR clones, 1044bp clones were isolated from all four Old World primate species. These clones have the same sequence as the larger product but, inferring a similar intron-exon arrangements and

boundaries as found in humans and chimpanzees, with an absent H2 exon. This H2 deletion was present in clones from PCR products of both IgD3 and IgD13, and IgD7 and IgD13 primer sets. In two of the three cynomolgus macaques examined, all of the clones sequenced had the H2 deletions. Conversely, the clones of the third cynomolgus macaque included the H2 exon. A single clone for each of the other species (baboon, rhesus macaque and sooty mangabey) was sequenced that lacked the H2. In addition to clones that were verified by sequencing, clones for each species with inserts of size corresponding to the H2 deletion product were observed on 1% agarose gels. No clones missing the H2 exon were observed for chimpanzee clones or human clones when the same primer sets were employed. Besides the two primer pairs described, we also designed a primer set (BGDF and BGDR) flanking the hinge to amplify small PCR fragments. These primers were tested with cDNA of two different baboons than those reported here and produced primarily fragments of the size expected for the IgD Δ H2 product when visualized as bands on an agarose gel. The bands for the product with a complete hinge were faint. These experiments likely under represent the full-length hinge product, because PCR amplification would favor the smaller IgD Δ H2 product, which was nearly half the size of the product with H2.

Dog IGHD

We performed reverse-transcription PCR on dog PBMC total RNA using primers K9IGHDF1 and K9IGHDB6 derived from sequences within a contig (NW_140211) that had a high percent identity with *IGHD* of other species. The resulting 1468nt product

consisted of seven exons (CH1, H1, H2, CH2, CH3, M1 and M2) encoding a 416 amino acids polypeptide (Figure 4.2), and a portion of the 3' untranslated region. Comparison with the genomic sequence shows the dog *IGHD* spans 8.25kb and all the introns follow the GT...AG rule with the possible exception of the H2/CH3 intron, which does not begin with GT in the contig sequence. The cDNA sequences agree with the dog contig for all but two nucleotides. Residues corresponding to human IgD CH1 positions 56 and 63 are methionine and glutamate in the contig, but are lysine and aspartate respectively in our clones. All cysteines that form intra- and inter-chain disulfide bonds in humans IgD heavy chains are conserved in the dog IgD heavy chain. An additional cysteine is present in the dog H2 at position 16. The predicted molecular weight of the unglycosylated transmembrane protein is 45.409 kDa, and without the transmembrane domain is 39.836kDa. There are four potential N-glycosylation sites in the dog IgD heavy chain. N-glycosylation sites at CH2 N66 and CH3 N100 are sites conserved with other mammalian IgDs. The N-glycosylation site at CH2 N4 is shared with artiodactyls and the N-glycosylation site at CH2 N87 is also present in cow and sheep. Dog IgD heavy chain amino acid identities with horse, pig, cow and sheep are similar to those seen between these species. CH1 and the hinge are least conserved, while CH2, CH3 and transmembrane domains are more conserved. Residues of the conserved antigen receptor transmembrane (CART) motif are present in the dog IgD transmembrane domain with the exception of T31, which is also absent in the other species of the Laurasiatheria group, i.e. pig, cow, sheep and horse (Campbell *et al.*, 1994). Dog IgD is most similar to horse IgD for CH1, hinge, CH2 and CH3 with percent identities of 40.2%, 37.3%, 57.8% and

67.3%, respectively. Dog IgD TM is most similar to that of pig and sheep with 62% identity to each. The percent identity of dog IgD CH1-CH3 with that of primates is 39% (human 39.3%, cynomolgus 38.7%, mangabey 38.6%, rhesus 38.7% and baboon 38.5%).

Phylogenetic analysis and comparative analysis of IgD heavy chains

To gain further insight into the evolution of *IGHD*, additional analysis of *IGHD* in mammals was performed. A phylogenetic tree was constructed from IgD CH1-CH3 of different mammals (Figure 4.3). The relationship between taxa corresponds to accepted phylogeny, with the exception of that of the Old World monkeys.

The human IgD H1 may have originated from IgM CH2 (Putnam *et al.*, 1981). Therefore, we compared the amino acid sequences from IgD H1 and IgM CH2 of different species. Human H1 is 35.3% identical to a portion of human IgM CH2 (R46-T79). The H1 of the chimpanzees and Old World monkeys have 32.4% identity with the same portion of IgM CH2, but the percent identity was lower when dog or mouse IgM CH2 was used for comparison. IgD H1 S8 and T26 in humans in this scenario would derive from IgM CH2 S53 and T71 respectively. However, with the exception of humans, IgD H1 S8 is only conserved in chimpanzees and IgD H1 T26 is replaced by an arginine in all the examined nonhuman primate species. IgD H1 A22 of hominoids is substituted with S22 in all Old World monkeys which aligns with the conserved IgM CH2 S67. IgD H1 of species other than primates have less than 17% amino acid identities with IgM CH2.

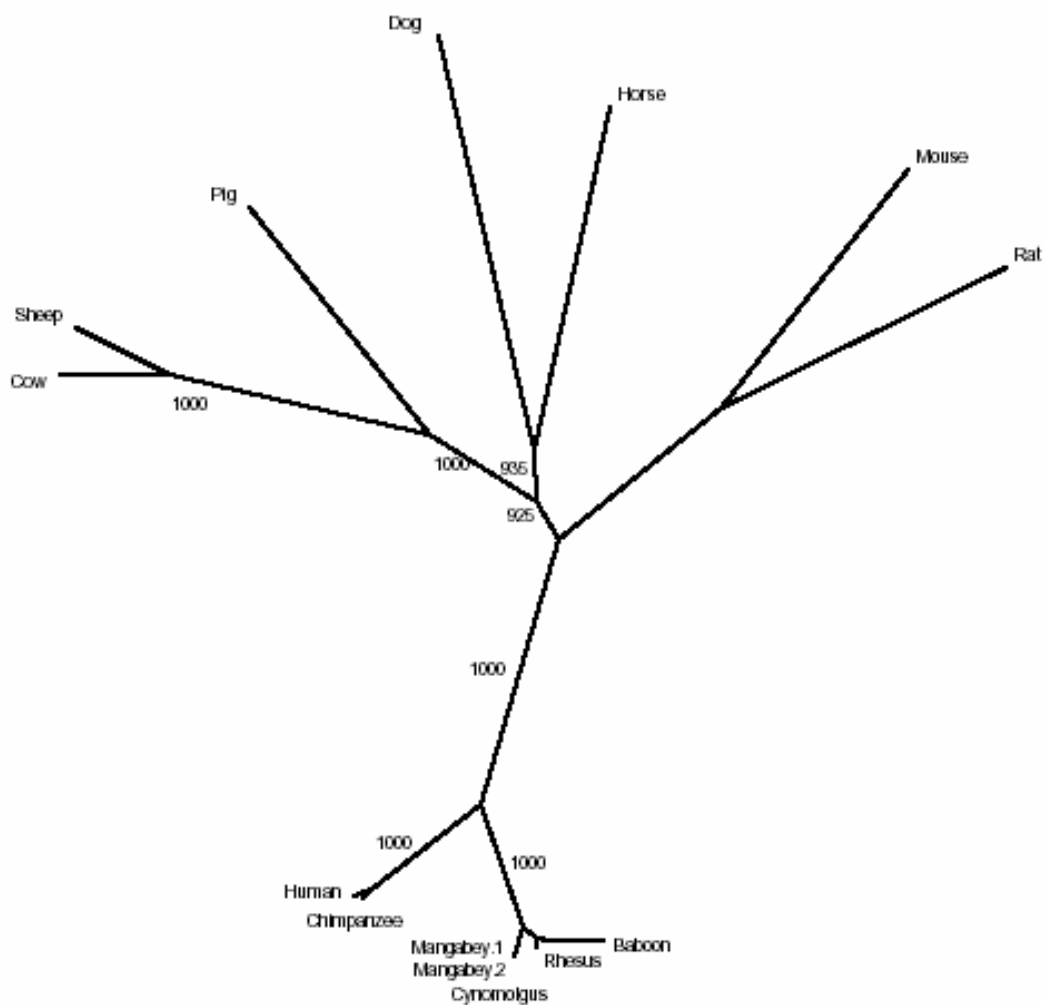


Figure 4.3. Phylogenetic relationships of mammalian IgD heavy chains. Neighbor joining tree constructed from the deduced amino acid sequences of mammalian IgD domains CH1-CH3 created using the CLUSTAL X method. Mangabey.1 and mangabey.2 represent two sequence variants. Accepted phylogenetic relationships between the different species are present in the tree except for Old World monkeys, which likely result from the high conservation of IgD within these species. The values shown represent the number of occurrences of branches over 1,000 bootstrap resamplings of the data sets.

In previously characterized *IGHD* sequences, different exons are conserved at different degrees. Table 4.1 summarizes the overall trends in deduced amino acid conservation observed between groups of mammals for the CH1-CH3 exons. As reported by others (Wagner *et al.*, 2004), the CH1 and the hinge exons are the least conserved. CH3 is the most conserved exon. In rodents, IgD CH2 is absent, but otherwise CH2 is conserved nearly as well as CH3 for other mammals. *IGHD* is well conserved in primates, both within the Old World monkey and hominoid groups, and between the two groups. Comparing Old World monkeys with hominoids, the IgD domain conservation is CH3>CH2>hinge>CH1. IgD CH3 is conserved 100% between baboon, mangabey and cynomolgus monkeys. The IgD hinge region of rhesus and mangabey is identical. The secretory tail (CH-S) of IgDs is poorly conserved between species. Of all the IgD domains, CH-S has the lowest percent identity between chimpanzees and humans (77.8%; chimpanzee CH-S: YVTDRGPVK versus human CH-S: YVTDHGPMK).

Discussion

Here, we have sequenced *IGHD* cDNA from five nonhuman primate species and from dog. Through comparison of these sequences and previously available *IGHD* sequences, we have examined issues related to the evolution of IgD and gained insight into structural IgD features that are most likely involved in functional properties. In agreement with what previously known about IgD, CH3 is the most conserved domain among the different species (Table 4.1). In contrast, the hinge region has diversified

extensively between different mammalian groups, but is well conserved within primates. Hence the hinge region may have evolved unique functional roles in primates.

Despite the importance of nonhuman primates in research, the immunoglobulin heavy chain constant regions of these species are only partially characterized. Sequences of genes are currently available for only a few antibody classes, mainly IgG and IgA, and are limited to a few species (Ueda and Kawamura, 1992; Calvas *et al.*, 1999; Scinicariello and Attanaio, 2001; Attanasio *et al.*, 2002; Scinicariello *et al.*, 2004). Furthermore, corresponding immunoglobulin Fc receptors, which are responsible for initiating cellular immune responses to antibody bound antigens, generally have not been characterized for nonhuman primates beyond identification by cross-reactive antibodies. The biology of humans and mice with regards to Fc receptors is often quite different (Takai, 2005). Therefore, nonhuman primates may provide useful alternative models to study antibody/Fc receptor interactions. Mice lack a homologue of the human IgA Fc receptor I (Takai, 2005), but homologues are present in macaques (Rogers *et al.*, 2004). In nonhuman primates, IgA is highly polymorphic and sequence differences from the human counterpart may result in modifications of their functional properties (Scinicariello and Attanasio, 2001; Scinicariello *et al.*, 2004). Overall, few studies have been performed to evaluate the functional properties of nonhuman primate antibodies, in part because of their incomplete molecular characterization.

Understanding of IgD has lagged compared to other immunoglobulin classes, but evidence has mounted over the past years for distinct roles played by IgD in the immune response. When immunized with a model antigen, the antibody repertoire of IgD⁺IgM⁻

mice differs from that of the IgD⁺IgM⁺ mice by VH gene usage, degree of affinity maturation, and reduced isotype switching to the IgG2a subclass (Han *et al.*, 2004). Both secretory and membrane bound IgD can bind to IgD receptors found on T cells of humans and mice (Amin *et al.*, 1991; Rudd *et al.*, 1995). In contrast to Fc receptors for IgE, IgG and IgA, these IgD receptors, identified over a decade ago, have remained poorly characterized making it difficult to assess their true function. In mice, antigen specific responses involving the cognate interactions of T and B cells are enhanced by the combined presence of IgD on B cells and up regulation of the IgD receptor on CD4⁺ T cells (Wu *et al.*, 1999). IgD can activate the alternate complement pathway (Spiegelberg, 1989). Despite the low concentration of IgD in normal sera, IgD serum concentrations are elevated under some circumstances (Preud'homme *et al.*, 2000). For example, increased serum IgD levels appear early and persist in HIV infections (Raiteri *et al.*, 1991). IgD levels also increase in humans with atopy (Peng *et al.*, 1991). Pathogen-specific IgD can be produced in response to infection (Preud'homme *et al.*, 2000). IgD makes up 3-10% of immunoglobulins in nasal, lacrimal and parotid glands, and in IgA deficient individuals IgD increases to 34-57% of the total immunoglobulins at these locations (Brandzaeg *et al.*, 1991). Elevated levels of serum IgD are characteristic of an autosomal recessive disorder, hyper IgD and periodic fever syndrome (HIDS), which is caused by mutations in the mevalonate kinase gene (Centola *et al.*, 1998). Although symptomatic attacks in HIDS patients do not correlate to IgD serum levels, attacks are marked by high levels of IL1, IL6 and TNF- α , the same cytokines induced by incubation of normal PBMC with IgD, suggesting a possible link between IgD and HIDS pathology (Drenth *et al.*, 1996).

Our findings show IgD structure of nonhuman primates and dog is similar to that found in human, horse and artiodactyls (cow and sheep). Three CH domains and a long hinge are present in all the examined primates and in dog. By contrast, mouse and rat IgD have no CH2, although a mouse pseudo-exon related to CH2 has been described (Mushinski *et al.*, 1980; Sire *et al.*, 1982; Richards *et al.*, 1983). Our results cannot establish whether or not the dog immunoglobulin identified by Yang *et al.* (1995) corresponds to the one that would be produced from dog *IGHD*; however, our data is compatible with this possibility. The calculated molecular weight of dog IgD CH1-CH3 domains is 40kDa. A complete IgD heavy chain including glycans at its four N-glycosylation sites, the heavy chain variable domain and a secretory tail (which we did not identify) would be expected to be of a greater molecular weight. This would be consistent with the reported molecular weight of 55kDa of the putative dog IgD heavy chain found in sera (Yang *et al.*, 1995).

N-glycosylation is an important feature of all immunoglobulin molecules including IgD. All the N-glycosylation sites found in humans are conserved in nonhuman primates. In humans, the N-glycosylation site at CH2 N66 is necessary for the association of IgD heavy chains to form a complete antibody and for secretion from the endoplasmic reticulum (Gala and Morrison, 2002). The N-glycosylation site at CH3 N49 is characteristic of all the examined primates and not found in IgD of the other species. *Cynomolgus* macaque IgD has additional N-glycosylation motifs present at CH1 N50 and CH2 N93. Mice possess an IgD receptor different from that of humans, which is expressed on CD4⁺ T cells and is specific for N-linked glycans on murine IgD (Adachi

and Ishizaka, 1986; Amin *et al.*, 1991). It is unknown if similar IgD receptors are present in other species, but possible N-glycan sites are present in dog IgD that might provide points of interaction with such an IgD receptor.

The structure of the immunoglobulin hinge regions is critical for their function; it gives antibodies the flexibility needed to bind antigen and provide sites of interaction with Fc receptors and complement. The IgD hinge is quite diverse in structure between species. Rodents have a shorter IgD hinge encoded by a single exon. The hinge regions of dog and chimpanzee are encoded by two exons. Old World monkey IgD hinge regions similarly have two distinct segments that are highly conserved with those of hominoids, including the large number of lysine and glutamate residues of the C-terminal portion. These charged residues may favor the formation of an α -helix structure and act to separate the two hinge segments by repulsion (Sun *et al.*, 2005). The IgD hinge region is the longest of that found in all human antibody isotypes and, besides the IgG3 hinge, is the only other immunoglobulin hinge encoded by multiple exons (Lefranc and Lefranc, 2001). The dog IgD hinge region is structurally distinct from the corresponding primate regions and is related more closely to that of ungulates; it does not have a highly charged second domain. The second half of the dog hinge region contains a cysteine which is at a conserved position with cysteines found in the hinge regions of sheep and cow. In the latter two species a second cysteine is also present in the IgD H2. It is possible that these cysteines in the IgD hinge regions of dogs, sheep, and cows are involved in forming inter-heavy chain disulphide bonds as described for the cysteines of IgG hinge regions.

Structurally, IgD and IgA1 hinges of humans share common features. IgA1 is the only other immunoglobulin with O-glycosylation in its upper hinge. As previously demonstrated for IgA, the flexible arms of IgD Fab can be separated by a wide angle thus resulting in an average antibody conformation similar to that of a T-shape in contrast to the more typical immunoglobulin Y-shape (Løset *et al.*, 2004; Sun *et al.*, 2005). Comparable to the hinge of IgD, IgA of different mammals is highly variable in length and amino acid sequence. IgD H2 is high repetitive. It has been suggested that the repetitive genetic structure of the IgA hinge region has led to its evolutionary instability (Flanagan *et al.*, 1984). Indeed, the hinge of two mouse species has diverged 25% in the mere 4-8 millions since the two species have separated (Osborne *et al.*, 1988). Old World monkeys, unlike humans, have only a single *IGHA* that encodes a short hinge more like the human IgA2 hinge without multiple O-glycosylation sites (Scinicariello *et al.*, 2004). The IgA hinge within rhesus macaques is highly polymorphic (Scinicariello *et al.*, 2004). By contrast, nonhuman primate IgD amino acid substitutions were found only in a sooty mangabey at two positions in CH2. More importantly, the hinge is highly conserved between primates; rhesus macaque and sooty mangabey IgD hinge regions are identical. Therefore, the hinge region of IgD appears to be less variable and evolutionarily more stable than the hinge region of IgA in nonhuman primates.

In humans, three glycine residues encoded at the start of H2 contribute to IgD hinge segmental flexibility (Sun *et al.*, 2005). Though conserved in chimpanzee, the glycines at H2 positions 3 and 4 are deleted in Old World monkeys. Loss of these glycines may be compensated for by a glycine dyad created by a glycine substitution next

to a conserved glycine in IgD H1 and by another glycine substitution in the middle of IgD H2. Besides being important for IgD receptor interactions (Rudd *et al.*, 1995), the O-glycans of the hinge contribute to structural rigidity (Sun *et al.*, 2005). We predict that IgD H1 O-glycosylation is reduced in nonhuman primates. At equivalent positions of the human O-glycan sites, IgD of chimpanzee has one substitution and IgD of Old World monkeys have three substitutions. In Old World monkeys, only IgD H1 S22 offers a potential O-glycosylation site not found in human IgD H1. Dog IgD hinge is not well conserved with that of primates. Hence, if present, dog IgD O-glycosylation is quite different.

In mangabey, baboon, rhesus macaque and cynomolgus macaque, we identified *IGHD* clones in which the hinge region that corresponds to the hominoid H2 exon was deleted. These transcripts (IgD Δ H2) may be the result of 1) an artifact of the reverse transcription PCR, 2) a polymorphism encoded by a second *IGHD* gene or allele or 3) alternative splicing events. Several lines of evidence argue against the first possibility. Under the same PCR conditions, and using the same primer sets, IgD Δ H2 transcripts were neither found in any of the chimpanzee clones, nor in control experiments using human RNA. The percentage of clones for each transcript varied between animals. Multiple primer pairs produced IgD Δ H2 clones. The deletion was always exact so that it maintained the reading frame. Finally, alignment of the hinge regions and CH3 were compared and no unusual sequences with high identity were found that might be conducive to PCR jumping. The latter two possible explanations remain to be tested and are not necessarily exclusive. For example, an allelic variant in the intron between H1

and H2 could alter splicing. Pig *IGHD* has an H2 exon that is spliced out because of a branchpoint mutation in the H1-H2 hinge intron (Zhao *et al.*, 2003). When a single T nucleotide is introduced into this sequence, pig *IGHD* transcripts include H2. Zhao *et al.* (2003) speculated that some transcripts with H2 might be produced normally in pigs, although none of their clones included the H2 exon. Future studies will be needed to test whether or not these primates express IgD without the H2 portion of the molecule, as such a difference may have profound effects on antigen binding properties.

The origin of the hinge in IgD is still an open question. Fish IgD have no hinge (Wilson *et al.*, 1997; Hordvik *et al.*, 1999; Stenvik and Jørgensen, 2000; Bengtén *et al.*, 2002; Hordvik, 2002; Hirono *et al.*, 2003; Saha *et al.*, 2004; Srisapoome *et al.*, 2004; Danilova *et al.*, 2005; Hansen *et al.*, 2005; Savan *et al.*, 2005). Putnam *et al.* (1981) have made the case that the human IgD N-terminal half of the hinge (H1) may have originated from a duplication of the *IGHM* CH2 domain, since human IgD H1 residues have a significant percent identity with those conserved between IgM CH2 of different species. Complementing this hypothesis is evidence that *IGHM* and *IGHD* are closely related and have exchanged genetic material in the past. Such evidence includes the probable origination of *IGHD* from an ancient duplication of *IGHM* (Zhao and Hammarström, 2003), and the demonstration that a duplicated *IGHM* CH1 replaced the original *IGHD* CH1 in artiodactyls (Zhao *et al.*, 2003). IgD H1 of primate appear to have a common origin, and nonhuman primate IgD H1 has a 32.4% identity with the same section of human IgM CH2 that has a 35.3% identity to human IgD H1. Such supports the hypothesis for a possible IgM CH2 origin of the primate IgD H1. This hypothesis does

not fit as well for species other than primates, which possess IgD hinges with less than 17% amino acid identities with IgM CH2. If the hypothesis is correct, then it would seem likely that IgD hinge regions arose in evolution three or more times, once for rodent hinge, once for primate hinge, and once for Laurasiatheria hinge. Additionally, the H2 of primates appears to be unique and may have yet another origin.

As anticipated on the basis of cross-reactivity obtained with anti-human antibodies, IgD deduced amino acid sequences of nonhuman primates are well conserved among the examined primates and humans. By contrast, dog IgD is not as well conserved, and it is not surprising that the available anti-IgD raised against IgD of other species do not cross-react with dog IgD. IgD CH1 and CH2 domains compared to CH3 and transmembrane domains are less conserved at the amino acid levels. This would seem to indicate that the IgD CH1 and CH2 domains are less critical for functional properties of IgD that are conserved across species, although these domains may have important species-specific functions. For example, in human IgD, these domains contain cysteines necessary for integrity of the quaternary structure. CH1 C15 forms bonds with the immunoglobulin light chains and CH2 C2 forms the only covalent bond between heavy chains. These cysteines are conserved in nonhuman primates and in dog.

IgD CH1 of artiodactyls shares a high degree of conservation with CH1 of IgM as a result of the *IGHM* CH1 having replaced the original *IGHD* CH1 (Zhao *et al.*, 2002; Zhao and Hammarström, 2003). Our data are in agreement with the hypothesis that the IgD CH1 replacement with IgM CH1 in artiodactyls was recent. Dog IgD CH1 is not highly conserved with the published dog IgM CH1 protein sequence (14.3% identity)

(McCumber and Capra, 1979). Horse IgD CH1 is not highly conserved with IgM CH1 (Wagner *et al.*, 2004). Therefore, the genetic event leading to the replacement of the original IgD CH1 in artiodactyls occurred after the evolution of carnivora, perissodactyla and cetartiodactyla as distinct phyla.

The *IGHD* M1 and M2 exons encoding the transmembrane domain are highly conserved between all species examined reflecting the importance of the transmembrane domain that they encode in establishing interactions with the B cell receptor associated signaling chains. As expected, dog *IGHD* maintains the M1 and M2 exon arrangement seen in other species. Presumably, this is also the case for chimpanzee *IGHD* for which we could not identify the M2 (because of the incomplete resolution of the chimpanzee contig sequence). Importantly, the dog and chimpanzee transmembranes domains contain the CART motif found in antigen receptors described by Campbell *et al.* (1994). This motif is involved in forming interactions with the B cell receptor signaling polypeptides (Campbell *et al.*, 1994).

Knowledge at the genetic level of IgD in species other than human and rodents has only begun to accumulate recently, and has demonstrated that IgD is widespread throughout vertebrates and is extremely diverse. Despite this diversity, IgD is surprisingly well conserved between nonhuman primates. In contrast to the view that IgD function as a BCR is redundant with IgM and that secreted IgD is unimportant, this high degree of conservation indicates that IgD may have valuable biological roles as yet unappreciated. Future studies should include the molecular characterization of IgD receptors and the determination of whether or not IgD receptors are a common feature of

mammals, thus leading to a clear definition of the roles that IgD plays in the immune response. The IgD sequences identified here would be valuable in such endeavors.

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CHAPTER 5

Immunoglobulin G (IgG) Fc Receptor III Homologues in Nonhuman Primate Species: Genetic Characterization and Ligand Interactions

Summary

Immunoglobulin Fc receptors are glycoproteins that bind to immune complexes through interactions with the Fc regions of specific antibody subclasses to initiate or inhibit the defense mechanisms of the leukocytes on which they are expressed. The human IgG low affinity Fc receptor III (CD16) can initiate phagocytosis and antibody-dependent cell-mediated cytotoxicity. CD16 has two isoforms, CD16a expressed on natural killer cells, monocytes as well as macrophages, and CD16b expressed on neutrophils. CD16a associates with either FcR γ or TCR ζ signal molecules to transduce activation signals. Although the development of therapeutic IgG molecules usually involves testing in nonhuman primates, the Fc receptors in these species have not been studied. Therefore, we have identified and characterized CD16 homologues in rhesus macaques, cynomolgus macaques, baboons and sooty mangabeys. Similar to humans, CD16 expression was detected on a lymphocyte subpopulation, on monocytes, and on neutrophils of sooty mangabeys. By contrast, CD16 was detected only on a lymphocyte subpopulation and on monocytes in macaques and baboons. In each nonhuman primate species, we identified a single CD16 gene encoding a protein at least 91% identical to human CD16a and a TCR ζ gene predicted to encode a protein with a conserved transmembrane domain necessary for CD16a association. A recombinant nonhuman

primate CD16 generated in HeLa cells interacted with human IgG1 and IgG2. By contrast, human CD16 binds to IgG1 and IgG3. Similar to human CD16, the monoclonal antibody 3G8 was able to block IgG binding to nonhuman primate CD16. Inhibition of nonhuman primate CD16 N-glycosylation enhanced IgG binding. Differences in interaction with IgG subclasses and in cells type expression should be considered when using these species for *in vivo* evaluation of therapeutic antibodies.

Introduction

Fc receptors are plasma membrane glycoproteins that bind to the Fc region of one or a few classes of antibodies. Cross-linking of antibody Fc receptors by antibody-opsonized antigen complexes initiates cellular immune responses including phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), respiratory burst, release of cytokines and inflammatory mediators, and antigen presentation (Ravetch and Bolland, 2001). Fc receptors are therefore crucial for the destruction and clearance of pathogens and tumors. Different Fc receptors with specificity for each of the five classes of antibodies (IgM, IgD, IgA, IgE and IgG) have been identified in mammals. Human IgG Fc receptors include Fc γ RI, Fc γ RII, and Fc γ RIII, which differ for cell type distributions and affinity for the four subclasses of human IgG (Ravetch and Bolland, 2001). Human Fc γ RIII, also known as CD16, is specific for IgG1 and IgG3 (Tamm and Schmidt, 1997).

Humans express two 97% identical Fc γ RIII isoforms, CD16a and CD16b, encoded by separate genes consisting of two immunoglobulin-like domains and a tail region linking the protein to the plasma membrane (Ravetch and Perussia, 1989; Gessner

et al., 1998). CD16a is expressed on monocyte subpopulations, macrophages, natural killer cells, select $\gamma\delta$ T cells, and can be induced on glomerular mesangial cells (Lanier *et al.*, 1985; Ravetch and Kinet, 1991; Radeke *et al.*, 1994; LaFont *et al.*, 2001). Because CD16a is the only Fc receptor expressed on natural killer cells and is responsible for IgG initiated ADCC, it has been called the ADCC receptor (Ahmad and Menezes, 1996; Mandelboim *et al.*, 1999). The CD16a complex consists of three polypeptide chains: one unique ligand binding chain and two signaling chains. The ligand binding chain (CD16a or Fc γ RIIIa) spans the plasma membrane and has a short cytoplasmic tail. In humans, the signaling chains of the complex are composed of either a homodimer or a heterodimer of FcR γ and TCR ζ that are required for efficient assembly, transport of the receptor from the endoplasmic reticulum to the plasma membrane and retention on the plasma membrane (Lanier *et al.*, 1989; Anderson, 1990; Gessner *et al.*, 1998). FcR γ is also a necessary component of the Fc ϵ RI and Fc γ RI complexes (Ra, 1989; Ravetch and Bolland, 2001). TCR ζ , expressed in T cells and natural killer cells, is also a component of the T cell receptor complex (Weissman *et al.*, 1989). In contrast to CD16a, CD16b is not associated with signaling chains. The CD16b isoform, exclusively expressed on neutrophils and eosinophils that have been exposed to IFN- γ (Hartnell *et al.*, 1992; Ravetch and Bolland, 2001), is linked to the outer plasma membrane by a glycosyl phosphatidylinositol (GPI) link, and modulates cellular responses through interactions with the other neutrophil Fc receptors (Scallon *et al.*, 1989; Jones and Brown, 1996). Association of the FcR γ signaling chain dimer with CD16a may contribute to a higher

ligand affinity of CD16a compared to CD16b (Miller *et al.*, 1996). No orthologues for CD16b have been identified in nonhuman species.

Nonhuman primates are widely used in biomedical research. The complex mechanisms of action and pharmacokinetics of therapeutic antibodies, usually IgG1 or IgG2 molecules, are routinely evaluated in these species (Yang *et al.*, 1999a; 1999b; Hahn *et al.*, 2001; Hinton *et al.*, 2003). Potential therapeutic cytokines and cytokine receptors are tested in nonhuman primates, and there is interest in extending these studies to immunoglobulin-cytokine fusion proteins as has been done in mice (Evans *et al.*, 1994; Munn *et al.*, 1996; Hérodin *et al.*, 2003). Macaques represent the accepted model for HIV vaccine development and AIDS pathogenesis (Hirsch and Lifson, 2000). CD16a is critical for natural killer cell targeted destruction of HIV infected cells through ADCC, although the relative importance of this mechanism *in vivo* is debated (Ahmad and Menezes, 1996). Recently, a small study in SIV-infected macaques indicated that sustained ADCC correlates with delayed onset of AIDS pathogenesis (Banks *et al.*, 2002). Xenograft rejection, also studied in nonhuman primates, is in part mediated through IgG directed ADCC via CD16 (Schaapherder *et al.*, 1994; Watier *et al.*, 1996; Cozzi *et al.*, 2005; Rood *et al.*, 2006). Nonhuman primates are widely used in testing monoclonal antibodies designed to prevent allograft rejection (Cosimi *et al.*, 1990; Kirk *et al.*, 1999; Kanmaz *et al.*, 2004; Kawai *et al.*, 2004; Koyama *et al.*, 2004; Schuler *et al.*, 2004).

CD16 homologues in nonhuman primates remain uncharacterized, and the premise that IgG/CD16 interactions in these species mimic those in humans has not been

fully evaluated. In the present study, we have identified nonhuman primate homologues of the human CD16 α chain and one of its associated signaling chains, TCR ζ , by sequencing these genes in four of the species most frequently used in biomedical research: rhesus macaque, cynomolgus macaque, baboon and sooty mangabey. Furthermore, we generated a recombinant nonhuman primate CD16 expressed on mammalian cells, and assessed its binding ability to the various IgG subclasses.

Materials and methods

Samples

Heparinized blood samples were collected from healthy animals of each of the following species: rhesus macaque (*Macaca mulatta*), cynomolgus macaque (*Macaca fascicularis*), baboon (*Papio hamadryas anubis*), and sooty mangabey (*Cercocebus torquatus*). Rhesus macaque and baboon samples were from animals housed at the Southwest National Primate Research Center San Antonio, TX. The samples from cynomolgus macaque and sooty mangabey were from animals housed at the Yerkes National Primate Research Center, Emory University, Atlanta, GA. Animal blood was collected under approval of the appropriate institutional review committees.

Determination of CD16 expression on blood leukocytes

Blood from 4 rhesus macaques, 7 cynomolgus macaques, 4 baboons and 4 sooty mangabeys was collected in EDTA Vacutainer® tubes (Becton, Dickinson and Co., San Jose, CA) by venipuncture under anesthesia. Leukocyte expression of CD16 on macaque

cells was analyzed by three-color flow cytometry analysis using cy-chrome conjugated anti-human CD16 (clone 3G8), phycoerythrin (PE)-conjugated anti-human CD89 (clone A59) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (clone SP34) (Becton, Dickinson and Co.). SimultestTM Control γ_1/γ_2 (Becton, Dickinson and Co.) was used to detect non-specific binding of mouse IgG to cells. Since cy-chrome conjugated anti-human CD16 was found to be poorly cross-reactive with baboon and sooty mangabey leukocytes, baboon and sooty mangabey leukocytes were analyzed by staining with anti-human PE conjugated CD16 PE (clone 3G8) and FITC conjugated anti-human CD3 (clone SP34). Additionally, sooty mangabey leukocytes were stained with CD89PE and CD16 FITC. Staining of whole blood was done using a standard procedure. Briefly, 100 μ l of blood was incubated with 20 μ l of each antibody in the dark at room temperature. Erythrocytes were lysed with 2ml of BD PharM Lyse (Becton, Dickinson and Co.), washed 3 times with PBS and fixed with 1% paraformaldehyde. 10000 events were counted by flow cytometry.

Amplification of nonhuman primate CD16 and TCR ζ cDNA

Total RNA was extracted from whole blood using the QIAamp RNA Blood Mini Kit (Qiagen, Inc., Valencia, CA), and reverse transcribed into cDNA using oligo d(T)17 primers, followed by primer extension with the AMV reverse transcriptase (Roche Diagnostic Corp., Indianapolis, IN). PCR amplification of the cDNA was performed with Expand High Fidelity polymerase (Roche Diagnostic Corp.) with the appropriate primer pair. Primers for amplification of primate CD16 were designed from the human sequence

assuming conserved homology between primates. The forward primer FCG3aF (5'-ATGTGGCAGCTGCTCCTCCCA-3') encodes the first 7 amino acids of CD16. The reverse primer FCG3aR (5'-TCATTTGTCTTGAGGGTCCTT-3') encodes the last 6 amino acids and the stop codon. TCR ζ and FcR γ primers were likewise designed. The forward primer TCRZ3F (5'-ATGAAGTGGGAAGGCGCTTTTCAC-3') encodes the first 7 amino acids of TCR ζ . The reverse primer TCRZ3B (5'-TTAGCGAGGGGGCA-3') encodes the last 3 amino acids and the stop codon of TCR ζ . The FcR γ forward primer FCRgamF (5'-ATGATTCCAGCAGTGGTCTTGCT-3') binds at the start codon, whereas the reverse primer FcRgam4 (5'-CTACTGTGGTGGTTTCTCATGCTTC-3') binds at stop codon and together amplify the complete gene. After initial denaturation at 95°C for 10 min, the cDNAs were amplified for 40 cycles, with each cycle consisting of 94°C for 1 min, 56°C for 1 min and 72° for 1 min 30 sec. A final step at 72°C for 10 min was used to ensure complete extension. For amplification of the signaling chains the step at 72°C was reduced to 30 sec. All reactions were performed in at least two independent reverse transcriptions PCR to verify product sequences. For CD16 of each nonhuman primate species 10 clones were sequenced from each of two independent PCR reactions.

Cloning of the amplified gene sequences

For cloning, 50 μ l of a reverse transcription PCR was run on a 1% agarose gel. The specific band of interest was excised from the gel and purified using a QIAquick® Gel Extraction Kit (QIAGEN, Inc.). The cDNA was ligated into pCR2.1 vector and transformed into Top10 *Escherichia coli* (Invitrogen Corp., Carlsbad, CA) followed by

expansion of select white colonies grown on media containing X-gal. Plasmid DNA was purified using a Wizard®plus Minipreps kit (Promega Corp., Madison, WI). The samples were screened on a 1% agarose gel after digestion with *EcoRI* to confirm the correct size of the DNA fragments. At least two clones from each PCR were sequenced. All DNA sequences were determined using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3100 Genetic Analyzer (PerkinElmer, Inc., Wellesley, MA). The forward and reverse M13 primers were used for sequencing. Overlapping regions were identified and sequences were edited using the MacVector software program (Accelrys Inc., San Diego, CA). Sequences were aligned with each other and other published sequence using the CLUSTAL function of the MEGALIGN part of the LASERGENE software package (DNASTAR Inc., Madison, WI).

Construction of nonhuman primate CD16 expression vectors

Full length nonhuman primate CD16 genes were first PCR amplified from cDNAs ligated into the pCR2.1 vector using forward and reverse primers FCG3FHinb (5'-AGATAAGCTTGATATGTGGCAGCTGCTCCTCCCA-3') and FCG3RBam (5'-TCTAGGATCCTCATTGTCTTGAGGGTCCTT-3'), which add *Hind III* and *BamHI* restriction sites 5' and 3' of the full length cDNA, respectively. CD16 fragments from clones with the correct sequence were released from vector by sequential digestion with *Hind III* and *BamHI*, cleaned up on a 1% agarose gel, and ligated into the *Hind III* and *BamHI* digested expression vector pcDNA3.1 (+) (Invitrogen Corp.) to create

pcCD16 vectors. CD16 expression vectors were then cloned into Top10 *E. coli* and colonies screened by sequencing with primers T7 and T7 reverse.

To improve the stability of the expression vectors in transfected cells, part of the pcDNA3.1 constructs were amplified (containing cDNA of nonhuman primate FcγIIIa, a cytomegalovirus promoter, a bovine growth hormone polyadenylation signal, and a fl origin) and inserted into the vector pLSXN (Becton, Dickinson and Co.), which contains long terminal repeats for integration into chromosomes. pLSXN contains a neomycin resistance gene, which allows for transfectant selection. Insertion of a pcDNA-CD16 fragment into pLSXN was carried out by amplifying with primers PcHp (5'-CTGCTGTAAACCGTTAGGGTTAGGCGTTTTGCG-3) and PcSa (5'-ACTTTGTCGACGCTCAGCGGCCGGCCATCGATCCACAGAATTAATTCGCGTT-3). The resulting fragment was then digested with *Hpa I* and *Sal I* and pLSXN digested with *Hpa I* and *Xho I*. The two fragments were then ligated together to form pLSXN-CD16.

Generation of recombinant CD16 by transfection of expression vectors into HeLa cells

Large quantities of vector for transfection were prepared using EndoFree® Plasmid Maxi kits (Qiagen Inc.). Twenty µg of expression vector was added to HeLa cells suspended in 250µl of Dulbecco's modified Eagle medium (DMEM) at 14×10^6 cells/ml. After ten min of incubation on ice, cells were electroporated by one pulse using the power supply set to 300V, 25mA, and 25W and the Electroporator II (Invitrogen Corp.) set to 1000µF and $\infty\Omega$, according to the manufacturer's recommendations. Cells

were then incubated at room temperature for ten min and grown in 10ml of DMEM 10% FCS in 5% CO₂ at 37°C. Antibiotic G418 (400µg/ml) was added to cell 72 hours post transfection to obtain stable transfectants.

Once cells recovered, an initial screen for successful transfections was carried out by flow cytometry. Adherent cells were removed from flasks with 90% confluent growth using 0.25% trypsin/2.21 EDTA in Hank's Balanced Saline Solution for 10 min. Cell number and viability was assessed using trypan blue exclusion on a hematocrit. PBS washed cells (0.5×10^6) were stained by incubation with 5µl of cross-reactive phycoerythrin-conjugated (PE) mouse anti-human CD16 clone 3G8 or mouse isotype control (Becton, Dickinson and Co.) for 30 min at 4° C. Unbound antibody was then removed with three washes of 200ul of PBS. Finally, cells were fixed with 200µl of 1% paraformaldehyde and analyzed using a FACSCalibur flow cytometer (Becton, Dickinson and Co.).

Clonal expansion

Selection and expansion of clones was performed following identification of successful transfections as indicated by positive stain for CD16 and a positive reverse transcription PCR for CD16. To isolate single clones, cells were diluted serially into 96 well microtiter plates. Cells were grown in 100µl of DMEM 10% FCS with G418 400µg/ml which was 50% fresh media and 50% conditioned media collected from flasks of untransfected HeLa cells and filtered with a 0.2µm filter. Each well was examined by

microscopy to identify wells with a single cell. Clones were subsequently expanded in wells of increasing size until cell numbers were sufficient to screen for CD16 expression.

Purification of nonhuman primate IgG

Nonhuman primate IgG was purified from serum from each species. Briefly, 200µl of serum diluted 1:1 in Pierce ImmunoPure® IgG binding buffer was incubated with 200µl ImmunoPure® Plus immobilized protein G for 30 min at room temperature (Pierce Biotechnology, Inc., Rockford, IL). The mix was then transferred to a 0.45µm filter tube and centrifuged for 2 min at 6g and the filtrate discarded. Following four washes with PBS at a (pH 7.2) and 10 min incubation at room temperature with Pierce ImmunoPure® IgG elution buffer, the tube was spun for 2 min and the filtrate collected. Finally, the filtrate was dialyzed against water using Spectral/Por® cellulose ester MWCO 50,000 (Spectrum Laboratories, Inc., Ft. Lauderdale, FL), first with two incubations at room temperature for 1 hour each and then overnight at 4°C with the buffer being changed each time. Purity of IgG was verified by a reducing SDS PAGE and the concentration checked by spectrophotometry at 280nm. IgG was frozen at -80°C prior to use.

IgG subclass ELISA

ELISA was used to verify the purity and identity of the human IgG myeloma proteins used in the CD16 binding assays. Microtiter plates were coated with human myeloma proteins (IgG1, IgG2, IgG3, or IgG4) (Binding Site Ltd., Birmingham, UK) or

purified total nonhuman primate IgG from the four species. After incubation at 4°C overnight, the plate was blocked with 5% FCS diluted in PBS at 37°C for 30 min. After washing, anti-human IgG1 (clone SG-16), IgG2 (clone HP-6014), IgG3 (clone HP-6050), or IgG4 (clone SK-44) were added to the plate and incubated for 1 hr at 37°C (Sigma-Aldrich Corp., St. Louis, MO). Following washing to remove unbound antibody, HRP-labeled goat anti-mouse IgG (H+L) was added to the plate and incubated for 1hr at 37°C (KPL, Inc., Gaithersburg, MD). The washed plate was developed by addition of ABTS/H₂O₂ followed by addition of stop solution, and its absorbance measured at 405nm using an automated Benchmark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA).

Immunoglobulin binding assay

Binding of immunoglobulin to CD16 was assessed by flow cytometry using antibodies that were heat aggregated at 63°C for 1hr. Human myeloma proteins IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM (Binding Site Ltd.) or IgE (Serotec Inc, Raleigh, NC) with immunoglobulin κ light chain as well as purified nonhuman primate IgG from different species were added to 0.5×10^6 cells at 20 μ g/ml and incubated for 1 hr at 4°C. PBS washed cells were then stained with 5 μ l of either FITC-conjugated mouse anti-human kappa or FITC-conjugated mouse IgG3 control for 30 min at 4°C (Invitrogen Corp.). In addition, in some experiments cells were stained using FITC-goat anti-human IgG (Invitrogen Corp.). For dual labeling experiments, mouse anti-human CD16 PE was added as well. Cells were washed and analyzed by flow cytometry as described above.

The ability of mouse monoclonal antibody 3G8 to block IgG binding was performed by first incubating harvested HeLa cells and mangabey CD16 expressing cells (0.5×10^6 cells/tube) with 20 μ l of 3G8 or control mouse myeloma IgG1 at different concentrations (0.5, 0.25, 0.125 and 0 mg/ml) for 30 min at 4°C. Afterwards cells were washed 3 times with PBS and IgG binding tests carried out as described above except that human myeloma antibodies were added at 40 μ g/ml. For N-glycosylation blocking experiments, tunicamycin was added to half of the cell cultures at 1 μ g/ml 30 hr prior to harvesting cells. Tunicamycin blocks the addition of N-glycans to proteins by inhibiting the transfer of N-acetylglucosamine-1-phosphate to dolicholmonophosphate.

Results

CD16 expression on macaque leukocytes

Although CD16 has been used as a marker of natural killer cells in nonhuman primates (Reimann *et al.*, 1994), CD16 expression on neutrophils has not been reported in these species. To determine whether or not CD16 is expressed on neutrophils in macaque species, whole blood from four rhesus and seven cynomolgus macaques was stained for flow cytometry analysis with anti-human CD16 Cy-Chrome, anti-human CD89 PE and anti-human CD3 FITC (Figure 5.1 C-H). Previously, it was determined that CD89 is expressed on macaque monocytes and granulocyte populations including neutrophils and eosinophils (Rogers *et al.*, 2004). Anti-human CD16 clone 3G8 was used because it binds to human CD16a natural killer cells on and human CD16b on neutrophils. CD16⁺

Figure 5.1. (Next page) CD16 expression on nonhuman primate leukocytes.

Representative two-color dot-plots of whole blood leukocytes from nonhuman primates stained for CD16 expression. A) forward scatter versus side scatter plot with leukocyte gates indicated, B) staining with mouse isotype controls, C-N) gated leukocyte populations (C, F, I and L granulocytes; D, G, J and M lymphocytes; E, H, K and N monocytes) stained for CD16 and either CD89 or CD3. CD16 expression was examined for rhesus macaques (C, D and E), cynomolgus macaques (F, G and H), baboons (I, J and K) and sooty mangabeys (L, M and N). At least 10000 cells were counted for all plots.

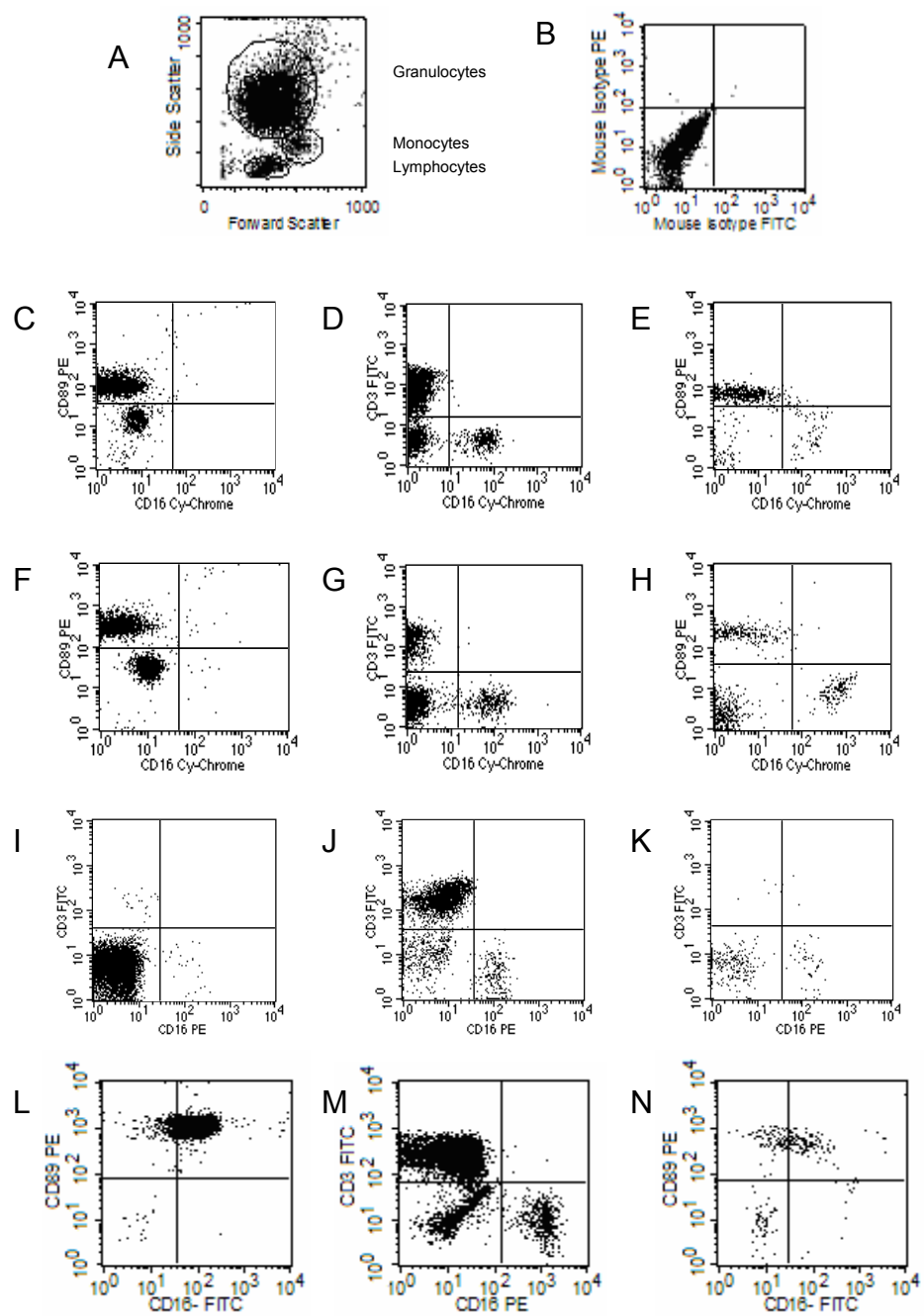


Figure 5.1.

leukocytes were $6.76 \pm 1.04\%$ (mean \pm standard deviation) for rhesus macaque and $11.02 \pm 3.55\%$ for cynomolgus macaque of the total peripheral blood leukocyte population. $CD89^+$ granulocytes (Figure 5.1 C and F) and $CD3^+$ lymphocytes (Figure 5.1 D and G) from both macaque species were $CD16^-$. $CD16^-$ cells constituted $11.75 \pm 4.28\%$ of rhesus macaque and $15.31 \pm 7.64\%$ of cynomolgus macaque lymphocytes (Figure 5.1 D and G). $14.67 \pm 4.66\%$ of rhesus macaque and $28.40 \pm 14.00\%$ of cynomolgus monocytes were $CD16^+$ (Figure 5.1 E and H). $CD89^+CD16^+$ monocytes accounted for less than 1% of total leukocytes and constituted $29.81 \pm 9.71\%$ of rhesus macaque and $8.84 \pm 8.26\%$ of cynomolgus macaque monocytes.

Three-color analysis was attempted for four baboons and four sooty mangabeys as done above for the macaques, but cy-chrome conjugated anti-human CD16 was not sufficiently cross-reactive. Whole blood from four baboons was stained with anti-human CD16 PE and anti-human CD3 FITC (Figure 5.1 I-K). In baboons, $CD16^+$ cells constituted $3.97 \pm 0.94\%$ of the peripheral blood leukocytes. Seventy percent of the $CD16^+$ cells were found within the lymphocyte gate and accounted for $11.92 \pm 6.95\%$ of the lymphocyte population (Figure 5.1 J). $CD16^+$ cells in baboons were $CD3^-$. Most of the remaining $CD16^+$ cells fell within the monocyte gate where 21.38 ± 13.71 of the cells were $CD16^+$ (Figure 5.1 K). $CD16^+$ cells made up $44.42 \pm 12.27\%$ of sooty mangabey peripheral blood leukocytes (Figure 5.1 L-N). Within the lymphocyte gate 6.70 ± 1.97 of the cells were $CD16^+CD3^-$ and $0.65 \pm 0.62\%$ were $CD16^+CD3^+$ (Figure 5.1 N). In the monocyte gate $25.61 \pm 13.43\%$ of the cells were $CD16^+$ ($21.85 \pm 12.86\%$ $CD16^+CD89^+$) (Figure 5.1

M). The majority of the sooty mangabey granulocytes were CD16⁺, with 72.51±14.29% staining CD89⁺CD16⁺ (Figure 5.1 L).

Cloning and sequencing of nonhuman primate CD16 genes

Cloned and sequenced cDNA of rhesus macaque, cynomolgus macaque, baboon and sooty mangabey was obtained through reverse transcription of total RNA isolated from whole blood followed by amplification performed using primers complementary to CD16 gene sequences located at the 5' and 3' ends of the coding region. The primers were designed to be complementary to the genes for both human CD16 isoforms. To verify that both human CD16 genes could be amplified with the primers, human CD16 cDNA was amplified, cloned and sequenced. All sequences from clones amplified using total RNA from human whole blood matched the CD16b isoform allele *FCGR3B*02* (GenBank accession number AJ581669), likely a result of CD16b transcripts outnumbering CD16a transcripts. Therefore, we stimulated THP-1 monocytic cells with PMA in the absence of estrogen to induce expression of CD16a. Amplification of cDNA from these cells yielded clones all matching a reported CD16a sequence (GenBank accession number X52645), thus validating our strategy to amplify both human CD16 isoforms. This strategy was then used to amplify CD16 from a single animal of each of four nonhuman primate species.

For all four nonhuman primate species, a single PCR band was present which represented a CD16 transcript with the complete reading frame encoding 254 amino acids, equivalent in size to human CD16. All nonhuman primate CD16 clones bore

greater sequence homology to human CD16a than CD16b. In nonhuman primates, residues at positions that differ between human CD16a and CD16b were conserved with human CD16a, including Gly147, Tyr158 and Phe203 (Figure 5.2). Phe203 is substituted by a Ser in CD16b and is critical to the expression of CD16b as a GPI linked protein on human neutrophils (Kurosaki and Ravetch, 1989). Single sequences were obtained for rhesus macaque, baboon and sooty mangabey CD16, whereas two sequences differing by a single amino acid (Lys25 or Arg25) were obtained for cynomolgus macaque CD16 (GenBank accession numbers DQ423376-DQ423380). All cysteines involved in forming intra-chain disulfide bonds in human CD16 are conserved in nonhuman primates (C47, C89, C128 and C172). Human CD16 (GenBank accession number CAA34755) has N-glycosylation motifs at Asn 56, Asn 63, Asn 92, Asn 180, and Asn 187. These motifs are conserved in nonhuman primate CD16 molecules, with the exception of Asn 92. An additional motif is present in nonhuman primates at Asn 82. Rhesus macaque and cynomolgus macaque CD16 amino acid sequences exhibit 91.7% and 91.3% identity to the human CD16a, respectively. The rhesus macaque CD16 amino acid sequence shows 99.6% identity to the corresponding cynomolgus macaque sequence. Baboon and sooty mangabey CD16 have identical amino acids sequences and share 92.5% identity with human CD16a, 98.4% identity with rhesus macaque and 98.0% identity with cynomolgus macaque. Therefore, CD16 sequences are highly conserved in nonhuman primates with only three residues distinguishing the African species (baboons and mangabeys) from the Asian species (macaques) (an Asp/Glu substitution at position 122, a Val/Met substitution at position 229 and a Ser/Arg substitution at position 238).

Figure 5.2. (Next page) Alignment of CD16 amino acid sequences of rhesus macaque, cynomolgus macaque, baboon, and sooty mangabey and humans. Nonhuman primate sequences were deduced from cDNA of whole blood. Human CD16a and CD16b sequences are from GenBank (accession numbers CAA34753 and J04162). Amino acid differences between human CD16a and nonhuman primate sequences are underlined. The mature peptide begins at residue 18 as indicated by the arrow. Two vertical arrows indicate the boundaries of exon S2, which is spliced out of one isolated rhesus macaque transcript. Potential N-glycosylation sites and cysteines involved in disulfide bonds are bolded. Shaded amino acids indicate amino acids in human CD16 that interact with human IgG1 (Sondermann *et al.*, 2000). Domains of the protein are indicated by arrows. The sequence of MafaCD16.2 was found to match an unpublished GenBank sequence (accession number AF485815). MafaCD16.1 is an experimentally isolated variant of CD16 differing at residue position 25. Hu: human; Soma: sooty mangabey; Paca: baboon; Mamu: rhesus macaque; Mafa: cynomolgus macaque. S1 and S2: signal peptide; Ig: immunoglobulin; Tm: transmembrane. Star indicates the amino acid critical for binding to monoclonal antibody 3G8.

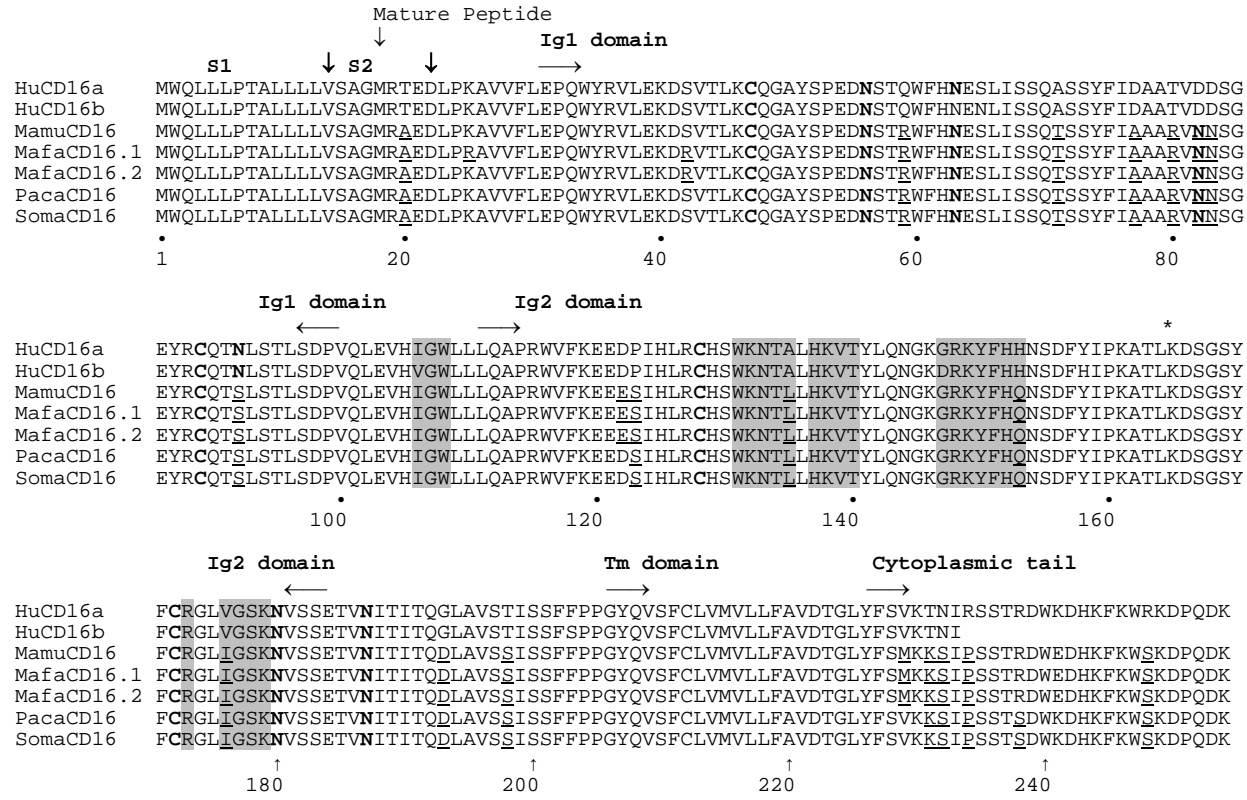


Figure 5.2.

Expression of sooty mangabey/baboon CD16 and identification of nonhuman primate TCR ζ chains

To characterize the ligand interactions of nonhuman primate CD16, the sooty mangabey gene was selected for expression in HeLa cells. The expression vector pLXSN-CD16 encoding mangabey CD16 was transfected into HeLa cells. These cells do not express Fc receptors and have been previously used to generate recombinant Fc receptors (Renedo *et al.*, 2001; Rogers *et al.*, 2004). Transcription of the introduced CD16 gene was determined by reverse transcription PCR. CD16 expression was assessed by staining with a PE conjugated anti-human CD16 and a mouse isotype control. Screening by reverse transcription PCR identified 9 clones which were positive for CD16 transcription. One clone resulted in high CD16 expression as determined by flow cytometry (mean fluorescence intensity MFI=1445.22) (Figure 5.3). Anti-CD16 staining was specific, since staining of untransfected HeLa cells (MFI= 8.12) and staining of the clone with a mouse isotype control (MFI =11.91) was low.

Mangabey CD16 expression was unexpected in HeLa cells without cotransfection with a signaling chain vector, because human CD16 requires coexpression of FcR γ or TCR ζ for efficient expression (Hibbs *et al.*, 1989; Kurosaki and Ravetch, 1989; Lanier *et al.*, 1989). To address whether or not HeLa cells express either signaling chain, reverse transcription PCR was performed. FcR γ transcripts were amplified successfully using RNA isolated from THP-1 cells (positive control), but not RNA isolated from HeLa cells. By contrast, reverse transcription PCR for TCR ζ yielded a band for the T cell line Hut-78 cells and a band of lesser intensity for HeLa cells. Cloning and sequencing of the band

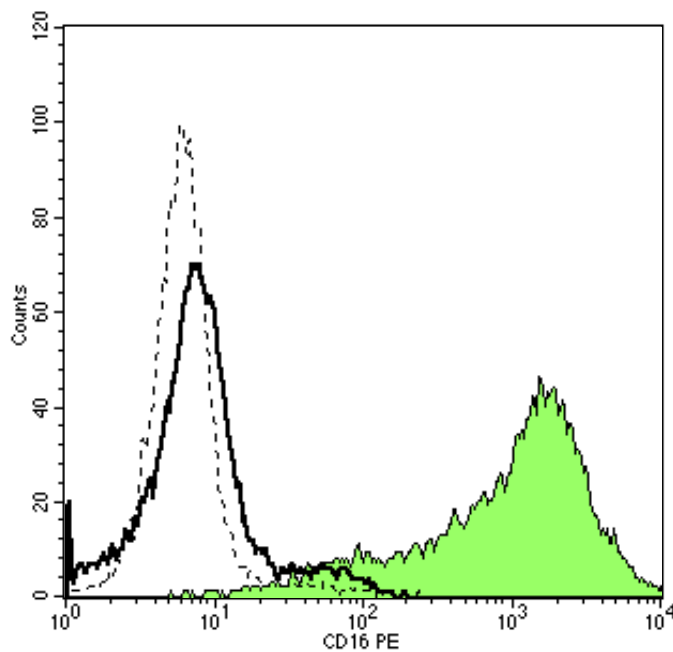


Figure 5.3. HeLa cells expressing high levels of recombinant sooty mangabey CD16. Histogram plot showing fluorescence of HeLa cells stained for the presence of recombinant mangabey with anti-human CD16 PE detected by flow cytometry. Filled curve-mangabey CD16 clone (MFI=1445.22), dotted line- HeLa cell control (MFI=8.21) and, solid line-clone stained with an antibody isotype control (MFI=11.91). 10000 events were counted per sample.

as well as a real time RT-PCR confirmed that TCR ζ is transcribed in HeLa cells albeit at levels lower than those found in Hut-78 cells. These results indicate that high levels of CD16 in HeLa cells may be permissible as a result of endogenous TCR ζ expression.

In contrast to human TCR ζ , mouse TCR ζ acts to down regulate CD16 as a result of a substitution of Leu 46 with Ile 46 in the transmembrane domain (Kurosaki *et al.*, 1991; Arase *et al.*, 2001). Therefore, TCR ζ cDNA from rhesus macaque, cynomolgus macaque, baboon and sooty mangabey was cloned and sequenced (Figure 5.4) (GenBank accession numbers DQ437667, and DQ437669, DQ437663 and DQ437665). Importantly, Leu 46 is conserved in Asian and African species. In baboons and sooty mangabeys the transmembrane domain and surrounding residues are completely conserved, whereas two substitutions (Ile41Leu and Val53Ala) are present in macaques. This conservation suggests that in mangabeys and boaboons CD16 and TCR ζ may associate similarly to what is observed in humans.

The percent identities of the deduced amino acid sequences compared with the human TCR ζ sequence for rhesus macaque, cynomolgus macaque, baboon and mangabey are 92.7%, 95.1%, 96.3% and 96.3%. The amino acid identity between the two macaque species is 98.2%. Baboon and mangabey deduced amino acid sequences are 100% identical, although there are differences at the nucleotide level. The baboon/mangabey amino acid sequence shares 97.0% identity with rhesus macaque and 98.8% identity with cynomolgus macaque. All TCR ζ chains from the nonhuman primates have an Asn and a Gln inserted between residues 131E and 132R of the corresponding human TCR ζ . As a result, nonhuman primate TCR ζ chains are two amino

	Signal Peptide	Extracellular		Cytoplasmic
		→ domain	→ Transmembrane domain	→ Tail
Human	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSA			
Baboon	MKWKALFTAAILQAQFPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSA			
Mangabey	MKWKALFTAAILQAQFPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSA			
Rhesus	MKWKALFTAAILQAQFPITEAQSFGLLDPKLCYLLDGILFLYGVILTALFLRAKFSRSA			
Cynomolgus	MKWKALFTAAILQAQFPITEAQSFGLLDPKLCYLLDGILFLYGVILTALFLRAKFSRSA			
Mouse	MKWKVSVLACILHVRFPGEAQSFGLLDPKLCYLLDGILFIYGVIIITALYLRAKFSRSA			
		#	\$!
Human	DAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRD <u>PEMGGK</u> <u>PQRRKN</u> <u>PQEGLYNELQKDK</u>			
Baboon	DAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRD <u>PEMGGK</u> <u>PQRRKN</u> <u>PQEGLYNALQKDK</u>			
Mangabey	DAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRD <u>PEMGGK</u> <u>PQRRKN</u> <u>PQEGLYNALQKDK</u>			
Rhesus	DAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRD <u>PEMGGK</u> <u>PQRRKN</u> <u>PQEGLYNALQKDK</u>			
Cynomolgus	DAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRD <u>PEMGGK</u> <u>PQRRKN</u> <u>PQEGLYNALQKDK</u>			
Mouse	ETAANLQDPNQL YNELNLGRREEYDVLEKKRARD <u>PEMGGK</u> <u>QRRRN</u> <u>PQEGVYNALQKDK</u>	1 st ITAM	*	2 nd ITAM
Human	<u>MAEAYSEIGMKGE</u> --RRRGK <u>GHDGLYQGLSTATKDTYDALHM</u> QALPPR			
Baboon	<u>MAEAYSEIGMKGENQRRRGK</u> GHDGLYQGLSTATKDTYDALHMQTLPPR			
Mangabey	<u>MAEAYSEIGMKGENQRRRGK</u> GHDGLYQGLSTATKDTYDALHMQTLPPR			
Rhesus	<u>MAEAYSEIGMKGENQRRRGK</u> GHDGLYQGLSTATKDTYDALHMQTLPPR			
Cynomolgus	<u>MAEAYSEIGMKGENQRRRGK</u> GHDGLYQGLSTATKDTYDALHMQTLPPR			
Mouse	<u>MAEAYSEIGTKGE</u> --RRRGK <u>GHDGLYQGLSTATKDTYDALHM</u> QTLAPR	+	+	3 rd ITAM

Figure 5.4. Alignment of TCR ζ deduced amino acid sequences. ITAM= immunoreceptor tyrosine activation motif (italics with conserved tyrosine and leucine/isoleucines bolded). Other features are underlined and indicated by symbols which appear below the position of the feature: # = cysteine involved in TCR ζ dimerization, \$ = aspartic acid which pairs with a charged residue of associated ligand binding chain (Lanier *et al.*, 1991), + = lysine and glycine residues in human peptide that bind GTP and GDP (Franco *et al.*, 1994), * = glutamine spliced into a variant that disrupts a G coupled protein binding motif, which includes 2 prolines prior and 1 after the glutamine (Atkinson *et al.*, 2003). != position 46 of leucine critical for human CD16 association (Kurosaki *et al.*, 1991). GenBank accession numbers for humans and mice are AL031733 and BC052824, respectively.

acids longer than their human counterpart (166 amino acids as compared to 164). In addition to the 164 amino acid TCR ζ polypeptide, humans also produce a 163 amino acid polypeptide that results from the splicing out of the codon encoding Gln 101 (Atkinson *et al.*, 2003). Splice variants were isolated for all four nonhuman primate species that were 165 amino acids long as a result of the same splicing event (GenBank accession numbers DQ437668, DQ437670, DQ437664 and DQ437666).

Mangabey CD16 binding to immunoglobulins

Binding assays were performed to determine the ability of mangabey CD16 to bind the different human and antibody subclasses and polyclonal nonhuman IgG. Heat aggregated myeloma proteins with an Ig κ light chain or polyclonal Ig were incubated with control HeLa cells or mangabey CD16 expressing cells followed by staining against bound antibody and analysis by flow cytometry.

Purity and identity of myeloma proteins was verified by ELISA using IgG subclass specific antibodies (data not shown). All tested subclass specific antibodies did not cross-react with purified nonhuman primate species' IgG. The staining for human IgG subclasses on mangabey CD16 expressing cells as measured by MFI was: IgG2 (131.78) and IgG1 (75.98) >> IgG3 (17.68) > IgG4 (10.05) (Figure 5.5). Staining of control HeLa cell for each subclass was as follows: IgG2 (10.75), IgG1 (10.16), IgG3 (14.08), and IgG4 (9.73). Staining of mangabey CD16 expressing cells using a FITC conjugated mouse antibody isotype control was negative (12.73). These results indicate that mangabey CD16 binds human IgG2 and IgG1 and only slightly binds IgG3, but not

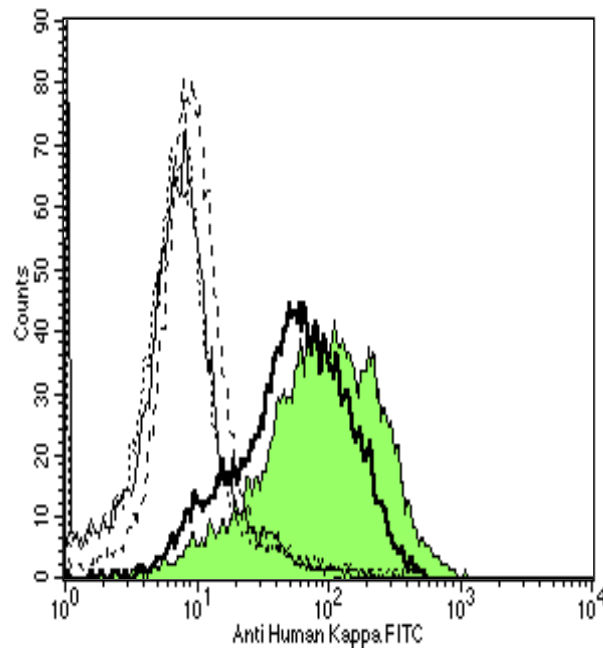


Figure 5.5. Binding of human IgG subclasses to recombinant mangabey CD16 expressed on HeLa cells. Human myeloma immunoglobulins of different subclasses all with Ig κ light chain were incubated with HeLa cells expressing mangabey CD16; bound immunoglobulins were detected with anti-human Ig κ FITC (IgG1: solid line MFI = 75.98, IgG2: filled MFI = 131.78, IgG3: dashed line MFI = 17.68, IgG4: thin line MFI = 10.05). As negative control, cells were stained with a mouse FITC isotype control (dotted line MFI = 12.73). 10000 cells were counted per sample. MFI= mean fluorescence intensity

IgG4. These results were independently verified using FITC conjugated goat anti-human immunoglobulin γ (data not shown). No staining for heat-aggregated human IgA1 (11.53), IgM (13.34), or IgE (7.75) was detected, nor for heat aggregated polyclonal mouse IgG (10.31). Polyclonal IgG from sooty mangabey and baboon bound to CD16 as detected by both FITC conjugated mouse anti-human immunoglobulin κ and FITC conjugated goat anti-human immunoglobulin γ (Figure 5.6). Similarly, binding was observed for purified polyclonal IgG of rhesus macaques and cynomolgus macaques.

IgG binding correlates with expression of sooty mangabey CD16 and is blocked by monoclonal antibody 3G8

Taking advantage of the variation in CD16 expression levels among different cells of the clone, IgG binding was correlated to CD16 expression. This was done by incubating HeLa cells expressing recombinant CD16 as well as untransfected HeLa cells with each of the four human IgG subclasses, followed by staining with PE conjugated anti-human CD16 clone 3G8 and FITC anti-human immunoglobulin κ . 3G8 is an antagonist of IgG binding to human CD16 (Hibbs *et al.*, 1994). As observed on dot plots of MFI for anti-human immunoglobulin κ versus MFI for anti-human CD16, there was a positive correlation between bound IgG and receptor level for the human subclasses IgG2 and IgG1 (Figure 5.7). As expected, no correlation was observed when using IgG3, IgG4 and IgA1. To ascertain whether or not a similar antagonist effect might exist for mangabey CD16, regression analysis was performed plotting anti-human immunoglobulin κ MFI versus anti-human CD16 MFI using different Ig subclasses.

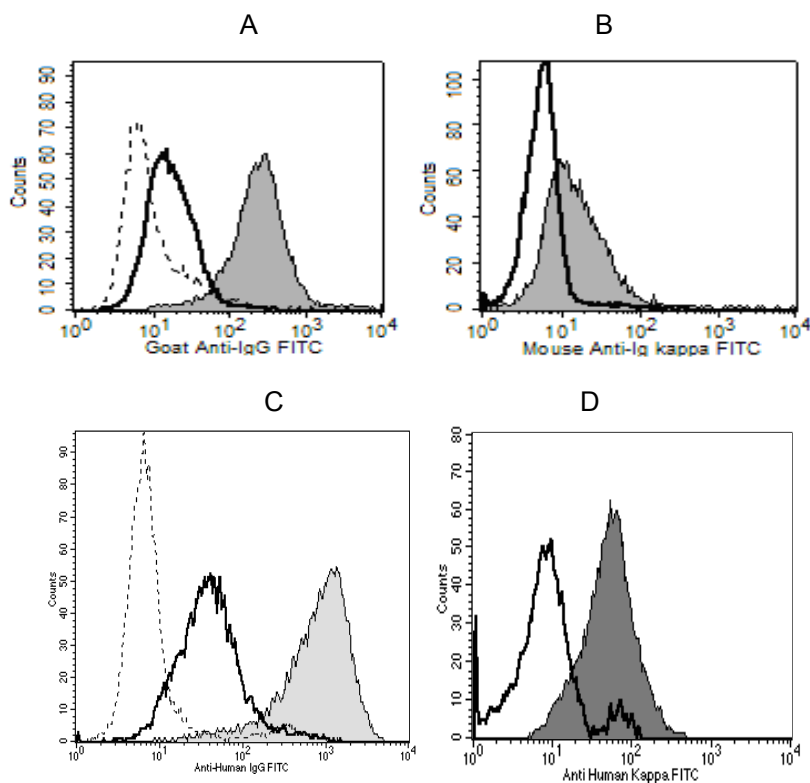


Figure 5.6. Binding of sooty mangabey (A & B) and baboon (C & D) IgG to HeLa cells expressing recombinant mangabey CD16. (A & C) detection of bound IgG with goat anti-human IgG FITC to mangabey CD16: filled (baboon MFI = 992.63, sooty mangabey MFI = 325.87), control HeLa cells: dotted line (baboon IgG MFI = 29.44, sooty mangabey IgG MFI = 37.17) and goat anti-human IgG bound in the absence of IgG to mangabey CD16: solid line (baboon MFI = 61.20, sooty mangabey MFI = 24.93). (B & D) detection of bound IgG with anti-human Ig κ FITC to mangabey CD16: filled (baboon IgG MFI = 62.04, sooty mangabey MFI = 24.46) and in the absence of IgG (baboon MFI = 7.67, sooty mangabey MFI = 7.69). MFI = Mean fluorescence intensity. 10000 events were counted per sample.

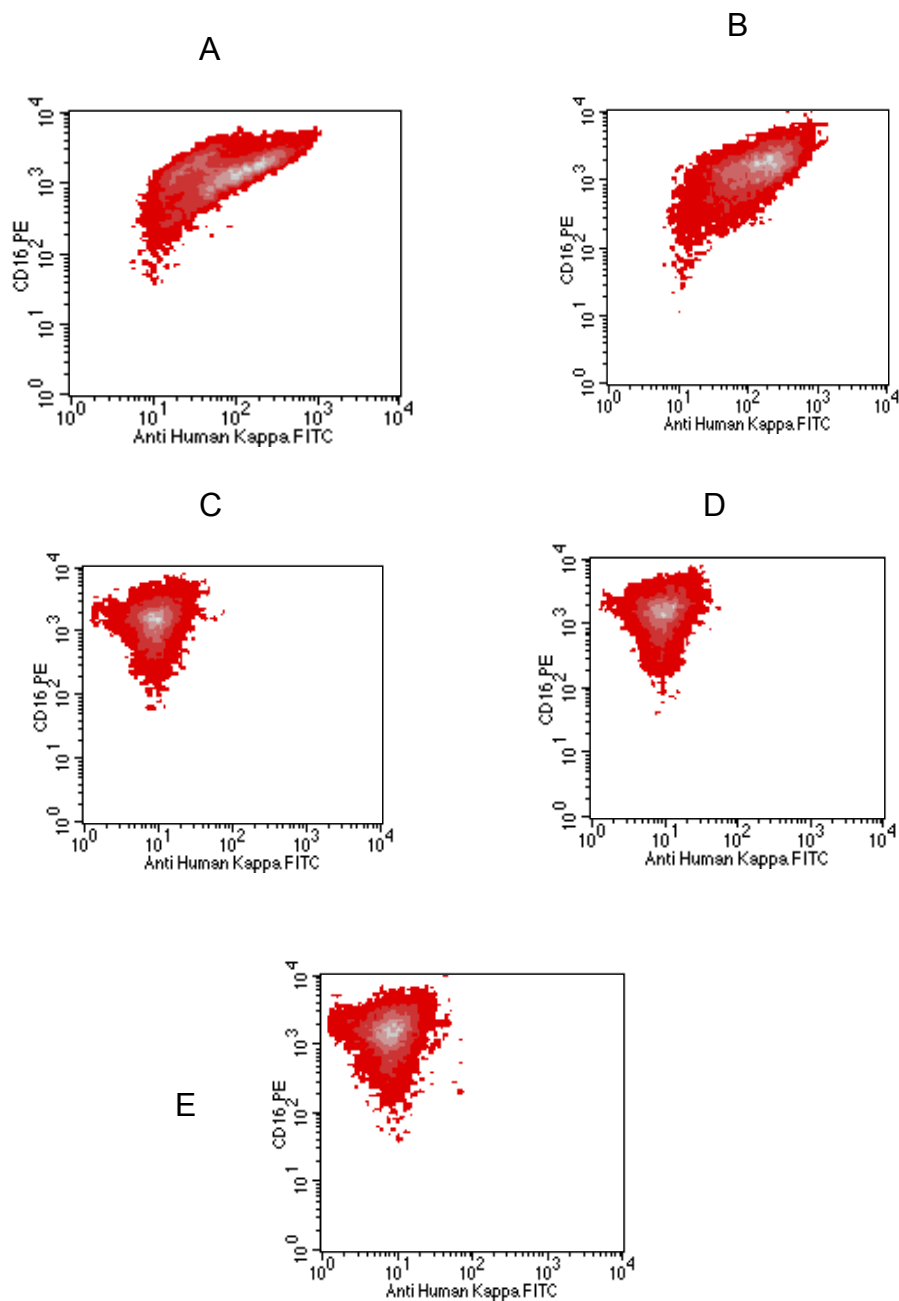


Figure 5.7. Binding of human IgG1 and IgG2 increases with increased expression of mangabey CD16 on HeLa cells. Following incubation with human myeloma immunoglobulins, which all have Ig κ light chains, cells expressing recombinant mangabey CD16 were stained with anti-human CD16 PE and anti-human Ig κ FITC. Density dot plots for IgG1 (a), IgG2 (b), IgG3 (c), IgG4 (d) and IgA1 (e). 1000 cells were counted by flow cytometry for each sample.

A significant negative correlation ($R^2 = -0.97$, $P = 0.006$) was present, thus indicating that IgG competes with the anti-human CD16 clone 3G8 for binding to mangabey CD16. These results also suggest that IgG did not completely saturate receptor binding sites at the concentration used. To verify that 3G8 blocks mangabey CD16 preventing binding of IgG, IgG binding experiments were repeated with cells that were first incubated with unlabeled 3G8 at different concentrations. Incubation of cells with 3G8 prior to addition of human IgG resulted in reduced binding of human IgG1 and IgG2 to mangabey CD16 (Figure 5.8). By contrast, incubation with a mouse antibody of irrelevant specificity prior to addition of human IgG did not alter the ability of mangabey CD16 to bind to human IgG1 and IgG2.

Effects of blocking N-glycosylation on the expression and ligand binding of CD16

Human and mangabey CD16 have conserved glycosylation motifs. Therefore, N-glycosylation of mangabey CD16 was blocked to assess the effects on IgG binding. Anti-human CD16 staining with monoclonal antibody 3G8 of tunicamycin treated cells decreased 15.43-17.47% compared to untreated cells. Previously, it has been shown that 3G8 binding to human CD16 is unaltered by glycosylation and that blocking N-glycosylation of human CD16 results in a modest decrease in its expression (Drescher *et al.*, 2003). Thus, the decrease in staining for mangabey CD16 likely represents a similar decrease in receptor expression. By contrast, staining for bound IgG increased for both IgG1 and IgG2 when N-glycosylation was blocked. Adjusting for the decrease in receptor expression, the increase in bound IgG1 and IgG2 ranged from 110% to 129.5% between

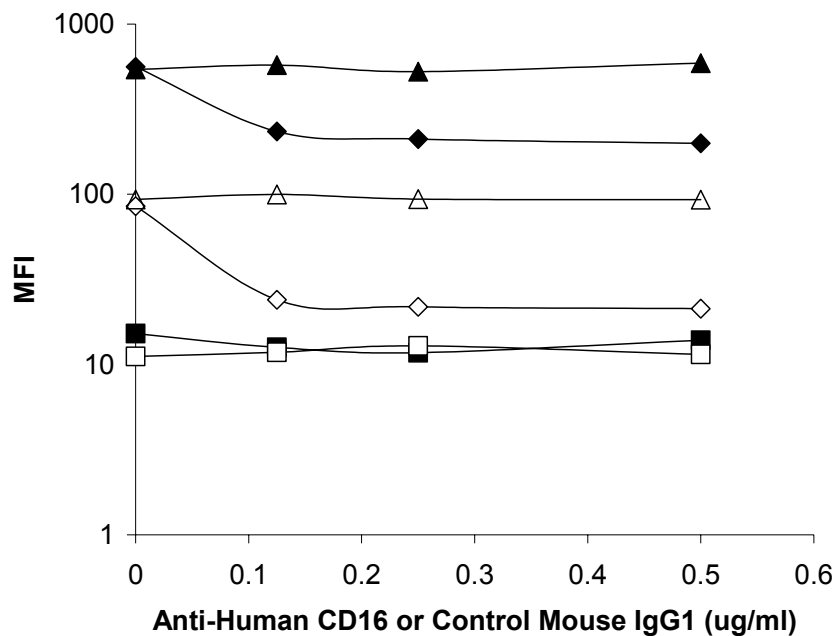


Figure 5.8. Monoclonal antibody 3G8 (Mouse Anti-human CD16) blocks binding of human IgG1 and IgG2 to recombinant mangabey CD16 expressed on HeLa cells. Different concentrations of 3G8 were incubated with HeLa cells expressing mangabey CD16 (diamonds) or with control HeLa cells (squares). Additionally mangabey CD16 expressing cells were incubated with an irrelevant mouse IgG1 control (triangles). Next, cells washed of unbound antibody were incubated with either human IgG1 (solid shapes) or human IgG2 (outlined shapes). Finally, washed cells were stained with FITC labeled anti-human Ig κ to detect bound human IgG. 10000 cells for each condition were read by flow cytometry and the mean fluorescence intensity (MFI) plotted against the concentration of monoclonal antibody 3G8 or mouse IgG1.

different experiments. The increase in IgG binding did not favor either isotype over the other. Staining for bound IgG3 and IgG4 was not significant regardless of N-glycosylation.

Discussion

Animal model studies of Fc receptor/antibody interactions have and will continue to yield useful insights into humoral immune responses, since only *in vivo* testing can mimic the complexities of the immune system. Mice have been used extensively for this purpose and have provided fundamental information about the function of Fc receptors. However, mice have no homologue of CD16b (Gessner *et al.*, 1998). Mice do possess CD16a, but this molecule does not appear to be a true orthologue of human CD16a because its extracellular region, responsible for binding to IgG, is more conserved with mouse Fc γ RII (Hughes, 1996). In addition, mouse Fc γ RIII is expressed on mast cells, whereas human mast cells do not express this receptor (Tkaczyk *et al.*, 2004). A second mouse receptor (CD16-2), which may be more homologous to human CD16a, has been recently identified (Mechetina *et al.*, 2002). However, this receptor has not yet been studied extensively. Nonhuman primates, which are widely used in immunological research and are more closely related to humans than mice, may be suitable models to study Fc receptors and related therapeutic strategies. Considering the importance of CD16 in immune responses and the use of nonhuman primates to evaluate human therapeutics which may bind CD16, we have identified and characterized CD16 ligand

binding chains and potentially associated signaling chains (TCR ζ) in rhesus macaques, cynomolgus macaques, baboons, and sooty mangabeys.

The presence of CD16 on nonhuman primate natural killer cells and monocytes was determined through staining of lymphocytes with cross-reactive anti-human CD16 (Reimann *et al.*, 1994; Munn *et al.*, 1996; Sopper *et al.*, 1997). Indeed, CD16 is recognized as one of the most reliable marker for phenotyping natural killer cells in macaques, while CD56, an informative marker for NK cells in humans, is not (Carter *et al.*, 1999). However, these studies do not provide information on the expression of CD16 on nonhuman primate neutrophils. Using flow cytometry and the anti-human CD16 clone 3G8, which cross-reacts with both human CD16 isoforms, we confirmed the presence of CD16 on nonhuman primate natural killer cells and monocytes in agreement with previous reports. We did not observe many CD3⁺CD16⁺ cells in any of the examined animals suggesting that most T cells in these nonhuman primate species do not usually express CD16. In humans, $\gamma\delta$ T cells can express CD16, but these are a minority of the T cell population and expression of CD16 on these cells may generally remain low until cells are properly stimulated (LaFont *et al.*, 2001). It remains to be determined whether or not nonhuman primates have $\gamma\delta$ T cells that can express CD16. In two of the sooty mangabeys a little over 1% of the cells in the lymphocyte gate stained CD3⁺CD16⁺. For all other animals examined the percentage of CD3⁺CD16⁺ cells was below 1% and comparable to the number of nonspecifically stained cells observed using mouse isotype control antibodies. Macaque and baboon granulocytes failed to stain positive for CD16. These results suggest the possibility that macaques and baboons may lack an orthologue

of human CD16b. Alternatively; any such orthologues may not be expressed at high levels on peripheral blood granulocytes in healthy animals or have diverged sufficiently so as to be undetectable by antibodies against the human receptor. In contrast, sooty mangabey granulocytes stained positive for CD16 on their cell surface.

Employing RT PCR capable of amplifying genes of both human CD16 isoforms, we identified a single gene homologue for each nonhuman species. These genes code for polypeptides that share at least 91% amino acid identity with human CD16, and more closely resemble human CD16a than CD16b. In particular, all genes from nonhuman primates possess a phenylalanine at position 203 like human CD16a. In human CD16b this residue is replaced with a serine that dictates the truncation and GPI linking of the protein to the plasma membrane (Kurosaki and Ravetch, 1989). The similarity of nonhuman primate CD16 with human CD16a is consistent with detection of CD16 on nonhuman primate NK cells and monocytes. We did not identify a nonhuman primate gene similar to the human CD16b gene. Differences in promoter regulation of the single CD16 gene identified in sooty mangabey may allow for its expression on granulocytes as well as monocytes and NK cells. The absence of CD16 on neutrophils from macaques and baboon suggests the absence of a CD16b gene homologue and requires further research. Humans may lack expression of CD16 on neutrophils without any apparent health problems (de Haas *et al.*, 1995). Thus, CD16b does not appear to be essential for effective immune responses, although it can modulate phagocytosis (Bredius *et al.*, 1994). Based on the high sequence homology of the two human CD16 genes, it appears

that the second gene arose recently from duplication of the original CD16 gene. This would explain why CD16b homologues have not been identified in other species.

CD16a is part of a larger complex that includes a signaling chain dimer with an immunoreceptor tyrosine activation motif. Human CD16a expression requires association with a signaling chain dimer of FcR γ , TCR ζ or a heterodimer of the two (Lanier *et al.*, 1989; Anderson, 1990; Gessner *et al.*, 1998). In mice, by contrast, increased expression of TCR ζ in NK cells down regulates CD16 expression (Arase *et al.*, 2001).

This species difference may be explained by differences in the TCR ζ transmembrane domain. Kurosaki *et al.* (1991) have shown that the Leu/Ile substitution at position 46 in the transmembrane domain of mouse TCR ζ causes the loss of the association of this signaling chain with CD16. We found that Leu 46 is conserved in all four nonhuman primate species. However, macaques exhibit substitutions that may influence the interactions with CD16. HeLa cells expressing TCR ζ , but not FcR γ , were able to express mangabey CD16. TCR ζ is normally only expressed in T cells and NK cells, because it is encoded by a gene controlled by a tissue-restricted promoter (Rellahan *et al.*, 1994). We also observed that when transient transfection was performed, mangabey and cynomolgus macaque CD16 expression was greater in a T cell line, HUT 78, than in HeLa cells that have lower levels of TCR ζ (results not shown). Thus, nonhuman primate CD16 may be positively regulated by TCR ζ , similar to human CD16.

Results from mutational analysis and antibody epitope mapping show that the CD16 membrane proximal immunoglobulin-like domain interacts with the lower hinge and CH2 domain of the IgG Fc region (Hibbs *et al.*, 1994; Shields *et al.*, 2001). Further

refinement of this model is based on the crystal structure of a human IgG1 fragment bound to soluble Fc gamma RIII (Sondermann *et al.* 2000). The crystal structure reveals that a single Fc γ RIII receptor binds asymmetrically to the two chain of a single IgG1 Fc fragment. Using the numbering of Sondermann *et al.* (IMGT unique numbering of CH2 immunoglobulin domain; LeFranc and LeFranc, 2001), IgG1 residues crucial for binding Fc γ RIII include Leu 234(4)-Ser 239(9), Asp 265(35), Ser 267 (37), Glu 269 (39), Ala 327 (97)-Ala 330 (100), and Ile 332 (102). In addition, the N-glycan at Asn 297 (67) is important (Sondermann *et al.*, 2000). Numbering from the start of the preprotein, the CD16 residues forming contact with IgG1 are Ile 106-Trp 108, Trp 131-Ala 135, His 137-Thr 140, Asp 147-His 153, Arg 173, and Val 176-Lys 179 (Sondermann *et al.*, 2000). The majority of the human CD16 residues important for binding IgG are conserved in nonhuman primate CD16 molecules. In all four nonhuman primate species Ala 135, His 153, and Val 176 are substituted with Leu, Glu, and Ile, respectively. In addition, Asp 147 is Gly in all nonhuman primate CD16 molecules as is found in human CD16a.

To assess nonhuman primate CD16/IgG interactions, we generated recombinant sooty mangabey CD16 in HeLa cells. The recombinant sooty mangabey CD16 was capable of binding to human IgG1 and IgG2, but not human IgG3 and IgG4. Despite the strong conservation of the mangabey CD16 sequence with the human CD16 sequence, mangabey and human CD16 differ in their ability to bind human IgG subclasses. Human CD16 binds to IgG1 and IgG3, but only weakly to IgG2 and IgG4 (Tamm and Schmidt, 1997). Mangabey CD16/IgG interactions are specific as indicated by the positive

correlation of cells labeled for both CD16 and bound IgG (Figure 5.7). Similarly, monoclonal antibody 3G8, an antagonist of IgG binding to human CD16, blocks binding to mangabey CD16 (Figure 5.8). These results indicate that the IgG binding site for mangabey CD16 is likely conserved with the human CD16 binding site as the sequence homology suggests, but only a few amino acid substitutions may be sufficient to allow for binding to IgG2. Failure to detect sooty mangabey CD16 binding to IgG3 could represent a limitation of the assay used. The myeloma IgG3 could have failed to form heat aggregates. However, under such circumstances, a low level of monomeric IgG3 binding to the receptor might then be anticipated. Alternatively, sooty mangabey CD16 may have low affinity for IgG3.

The IgG1 motif LLGGP located in the lower hinge is particularly important for binding to CD16 (Chappel *et al.*, 1991; Tamm and Schmidt, 1997). In IgG2 this motif is replaced by VAGP. Mutational analysis shows that the entire IgG2 lower hinge motif must be replaced with that of the IgG1 motif to allow binding to CD16. Mutations of IgG1 in its motif are detrimental to binding affinity (Chappel *et al.*, 1991). The motif LLGGP is encoded by all four IgG subclasses genes in baboons, rhesus macaques, cynomolgus macaques, pigtail macaques and sooty mangabeys with the exception of baboon IgG2 and pigtail macaque IgG4 (Attanasio *et al.*, 2002; Scinicariello *et al.*, 2004; and our unpublished results). Here, we have shown that recombinant mangabey CD16 is capable of binding to rhesus macaque, cynomolgus macaque, baboon and sooty mangabey IgG. Currently, purified nonhuman primate IgG1, IgG2, IgG3 and IgG4 are not available and therefore could not be tested in binding assays. Based on the

conservation of the IgG1 motif in most of these IgG molecules, it is expected that all four subclasses bind CD16. However, IgG/CD16 interactions have been shown to be complex and involve residues outside of the IgG lower hinge (Shields *et al.*, 2001). Radaev and Sun (2001) found that small peptides with the sequences matching those of the lower hinge of human IgG1, IgG2 and IgG4 all have similar affinities for human CD16 and concluded that additional IgG features, such as hinge length, are important. Human IgG3 contains the LLGGP motif, yet we did not detect its binding to mangabey CD16. Therefore mangabey CD16/IgG interactions are likely to also involve additional IgG subclass differences.

The N-glycan of human CD16 at Asn 180 is in close proximity of other residues that interact with the Fc fragment of IgG1 (Sondermann *et al.*, 2000). Glycosylation inhibition and mutation of Asn 180 to Glu results in increased affinity of CD16 for monomeric IgG, whereas mutations at other CD16 N-glycan sites have no effect (Drescher *et al.*, 2003). CD16a of monocytes, macrophages and NK cells have different affinities for IgG as a result of differential glycosylation (Edberg and Kimberly, 1997). Hence, cells can modulate binding to IgG through modification of the Asn 180 N-glycan. CD16 of nonhuman primates have the Asn 180 glycosylation motif. Inhibition of N-glycosylation with tunicamycin resulted in a modest increase of human IgG binding to mangabey CD16, just as is reported for human CD16 (Drescher *et al.*, 2003). As is the case for other molecules, CD16 glycosylation may be altered during inflammation to modulate affinity to IgG and hence activation through CD16 (Drescher *et al.*, 2003). Our data support the possibility that such a mechanism is also possible in nonhuman primates,

although it is unclear how important this would be in the context of the types of increased binding affinity we observed. Testing of this hypothesis will require examination of CD16 affinity and glycosylation changes under inflammatory conditions which would be more physiologically relevant than a total inhibition of glycosylation.

The extensive use of nonhuman primates in immunological studies makes it important to understand how their antibody responses compare to those of humans. To achieve this goal it is not only vital to study nonhuman primate antibodies directly, but also to understand how they interact with the various components of the immune system. Therapeutics that may directly interact with Fc receptors include monoclonal antibodies, immunoglobulin fusion proteins as well as small drugs being developed to inhibit Fc receptor function (Radaev and Sun, 2001). Our results are therefore critical for the proper interpretation of results from studies performed in nonhuman primates.

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Epilogue: Nonhuman Primate CD16 and Testing of Therapeutic Antibodies

Does the IgG Fc receptor III (CD16) play a role in determining the efficacy of therapeutic antibodies and other therapeutics? How would the human/nonhuman primate differences in CD16 expression profiles and binding characteristics influence testing of therapeutics in nonhuman primates?

Many therapeutic antibodies and other therapeutics containing the Fc region from antibodies are either approved for use or are being developed for use in humans for sundry medical conditions. Use of therapeutic antibodies is rapidly increasing with 19 FDA approved molecules and over 150 in development (Kim *et al.*, 2005). These molecules primarily are of the IgG isotype. Therefore, engineering and evaluation of these molecules must take into account potential interactions with CD16. In some clinical applications it is desirable to engage Fc receptor induced cellular effector mechanisms, for example when trying to target ADCC to cancer cells (Carton *et al.*, 2004), whereas in others it is preferable that Fc receptors not be engaged, for example when using anti-CD3 monoclonal antibodies to prevent transplant rejection (Cole *et al.*, 1997; Li *et al.*, 2005). Anti-tumor therapeutics are usually IgG1 molecules and thus bind to Fc receptors (Carton *et al.*, 2004). By contrast, IgG2 is a preferred subclass for anti-CD3 monoclonal antibodies (Cole *et al.*, 1997). Clearly, the IgG subclass of each potential therapeutic immunoglobulin must be selected on the basis of its ability to engage Fc receptors. Indeed, understanding IgG/CD16 interactions may improve the prognostic value of monoclonal antibody treatment. Multiple alleles exist for both isoforms of human CD16.

CD16a isoforms have either a valine or a phenylalanine at position 158, the former having higher affinity for IgG (Koene *et al.*, 1997). Therapeutic success rates are higher in individuals homozygous for the CD16 valine 158 allele receiving rituximab for follicular lymphoma or Waldenstrom's macroglobulinemia compared to individuals either heterozygous or homozygous for the phenylalanine 158 allele who receive similar treatment (Carton *et al.*, 2004; Weng and Levy, 2003; Treon, 2005). Immunoglobulin-fusion molecules, a class of therapeutics, typically consist of a biological active molecule (such as a cytokine or cytokine receptor) attached to the IgG Fc portion which includes the hinge region and the CH2 and CH3 domains (Kim *et al.*, 1998). Incorporation of the immunoglobulin Fc in these molecules has the advantage of extending their half-life and allowing for multimeric presentation. However, often it is desirable that these molecules not interact with IgG Fc receptors, including CD16, as such interactions would result in unwanted biological activities (Kim *et al.*, 1998). Other therapeutics being considered are peptidyl inhibitors that mimic IgG to block IgG receptors including CD16. These molecules would be potentially beneficial for treating autoimmune diseases, as for example rheumatoid arthritis, which are triggered by autoantibodies activating inflammatory cellular responses through Fc receptors (Radaev and Sun, 2001).

We found that CD16 in nonhuman primates is expressed on some cell types in common with those on which human CD16 is expressed, and nonhuman primate CD16 homologues do not always interact with the same IgG subclasses as human CD16. It would be imperative to consider these differences in monoclonal antibody safety studies in nonhuman primates. For example, studies of IgG2 monoclonal antibodies in these

animals may over estimate toxicity, because IgG2 may trigger CD16 directed NK cell ADCC responses that would not occur in humans (where IgG2 does not interact with CD16). On the other hand, neutrophil responses to IgG1 would also differ. In baboons and macaques CD16 seems to be absent from neutrophils. Therefore, IgG1 antibodies might trigger immune responses in human neutrophils that would not be uncovered from testing in nonhuman primates. While expression of CD16 on neutrophils of sooty mangabeys is more similar to what observed in humans, our data suggest that the mangabey receptor may be similar to the human isoform found on other cell types (CD16a) than the isoform found on human neutrophils (CD16b). These isoform differ in the transmembrane and cytoplasmic regions, hence in cell signaling capacities. Therefore, sooty mangabey and human IgG activation of neutrophils through CD16 is likely to be different.

CHAPTER 6

Conclusions

Our defenses against pathogens and malignancies comprise a complex array of interacting cells and molecules that make up our immune system. At the beginning of vertebrate evolution, the adaptive portion of the immune system budded from the older innate immune system and diversified. Antibodies, the quintessential component of the adaptive humoral immune system, act as adaptor molecules that link targets of the immune system to immune effector responses. As previously discussed, antibodies are highly diverse and are divided into classes and subclasses characterized by different functional properties, which result from differences in the structure of the constant regions. These functional properties are still not completely defined. To fully understand antibody function, it is necessary to characterize the other components of the immune system with which antibodies interact. Because the immune system of nonhuman primates most closely resembles that of humans, and because nonhuman primates are widely used for biomedical research, we characterized immunoglobulins and immunoglobulin Fc receptors in these species.

In mammals there are five classes of antibodies (IgM, IgD, IgG, IgA, and IgE), each of which is represented in humans. We have studied selected aspects of three classes of antibodies: IgA, IgD and IgG. IgA, the antibody class produced most copiously, is dominant in the mucosae, where it represents the frontline defense against pathogens, and is a major component of sera. IgD, along with IgM, is responsible for the initial

recognition of antigens and activation of B cells in adaptive humoral immune responses as part of the B cell receptor. IgG is the most abundant antibody in sera and is recognized as the most important antibody for systemic adaptive immunity. The characterization of structure/function relationships related to these classes of antibodies as well as the characterization of differences between species, especially humans and nonhuman primates, is incomplete. In this study, we have identified and characterized nonhuman primate IgD. In addition, we have extended our previous work on nonhuman primate IgA and IgG by defining the interactions of these two immunoglobulin isotypes with their specific Fc receptors.

While the role of IgA at mucosal surfaces is partially understood, the role of IgA in sera is mostly undefined. In the last decade, the discovery and characterization of a human IgA Fc receptor (CD89) has provided a context to understand the functions of serum IgA. Early studies of this receptor suggested its participation in activating cellular effector functions, including antibody-dependent cell-mediated cytotoxicity, phagocytosis and respiratory burst, as well as release of cytokines and inflammatory mediators. Results from recent studies point out that CD89 can also provide down regulation and activation signals to other Fc receptors, helping to explain the anti-inflammatory properties of IgA. Because of the lack of a reliable *in vivo* model system, research on IgA/CD89 interactions has been limited. At the onset of this project, no animal homologues of CD89 were known, despite extensive searches performed in mice (it is now established that mice most likely have lost the CD89 gene). Thus, we have characterized CD89 homologues in rhesus macaques, cynomolgus macaques, baboons and sooty mangabeys.

Nonhuman primate and human CD89 molecules are expressed on the same cell types and are highly conserved with each other (Figure 6.1). Furthermore, similar to what is known for humans, the four nonhuman primate species produce several different CD89 splice variants. By contrast, CD89 splice variants have not been identified in other mammalian species. By generating and using in binding studies recombinant rhesus macaque CD89 expressed on the cell surface of HeLa cells, we have determined that rhesus macaque CD89 can bind both human IgA subclasses and that its expression is dependent on N-glycosylation (Figure 6.1). Recombinant rhesus macaque IgA generated in our laboratory is also able to bind rhesus macaque CD89. These results indicate that IgA function is conserved between humans and nonhuman primates, thus validating the use of these animal models to study the functional properties of IgA *in vivo*.

IgD has been a neglected class of antibodies and little is known about its functions outside of its role as a B cell receptor. Because of its apparent redundancy with IgM, even the B cell receptor related function of IgD has been treated dismissively until recently. IgD is also present as a secreted protein in serum, although it is only found at comparatively low concentrations under most physiological conditions. Although IgD receptors were described in mice and humans over a decade ago, they remain poorly characterized. In stark contrast, important advances have been made in the characterization of Fc receptors for IgG, IgE and IgA. The faltering progress on IgD receptors has engendered a degree of uncertainty over the true function of these receptors. Nonetheless, there has recently been a resurgence of interest in IgD. IgD previously was

Table 6.1. Human and nonhuman primate expression of CD89 and CD16 on different leukocyte populations and antibody subclasses binding patterns of each receptor. NT not tested, hu: human, rh: rhesus macaque, NHP: nonhuman primate.

		Human	Rhesus Macaque	Cynomolgus macaque	Baboon	Sooty Mangabey
CD89	Lymphocytes	-	-	-	-	-
	Monocytes	+	+	+	+	+
	Granulocytes	+	+	+	+	+
	Antibody Subclasses	huIgA ₁ , huIgA ₂	huIgA ₁ , huIgA ₂ , rhIgA	NT	NT	NT
CD16	Lymphocytes	NK cells, $\gamma\delta$ T cells	NK cells	NK cells	NK cells	NK cells
	Monocytes	+	+	+	+	+
	Granulocytes	+	-	-	-	+
	Antibody Subclasses	huIgG ₁ , huIgG ₃	NT	NT	huIgG ₁ , huIgG ₂ , NHPIgG	huIgG ₁ , huIgG ₂ , NHPIgG

thought to be expressed in only a very limited number of mammals. The recent discovery of IgD in additional species has sharply challenged this view. We have characterized IgD in chimpanzees, rhesus macaques, cynomolgus macaques, baboons, sooty mangabeys and dogs, thus contributing information that will aid to shed light on IgD functions. IgD is expressed at high levels in certain pathogenic conditions including myeloma, hyper IgD and periodic fever syndrome, atopy, and HIV infection. Characterization of IgD in macaques, the accepted model for HIV, will make it possible to better explore the significance of elevated IgD in AIDS patients. It is our hope and intent that the characterization of nonhuman primate IgD be used to identify and characterize the IgD receptor. Only nonhuman primate IgD hinge regions are structurally similar to the corresponding human region, which is known to be responsible for interactions with the human IgD receptor. Structural similarities exist between the hinge region of nonhuman primate IgD and IgA, including a repetitive sequence which for IgA has been hypothesized to contribute to genetic instability. Sequence information on nonhuman primate IgD allows us to address this hypothesis. Our laboratory has previously found that IgA is highly polymorphic in nonhuman primates, showing that there is sufficient evolutionary human-nonhuman primate separation to allow for diversification of the IgD hinge. The strong conservation of the human-nonhuman primate IgD hinge region does not support the hypothesis that a repetitive genetic sequence is the most important factor influencing the diversity of the hinge region. Other factors that may have influenced hinge region diversity include selective pressure due to the existence of bacterial

proteases that cleave IgA molecules at the hinge region and possibly important conserved functions of the IgD hinge region, as for example interactions with an IgD receptor.

In humans, two genes encode the two isoforms of the IgG Fc receptor III (CD16). These isoforms differ from each other by only 3% in amino acid sequence, but are functionally distinct as a result of differences in cell type distribution and in how they are anchored to the plasma membrane. CD16a is present on monocytes, macrophages, and natural killer cells, contains a complete transmembrane domain that associates with a signaling chain, and is essential for directing natural killer cells antibody-dependent cell-mediated cytotoxicity. CD16b is expressed on neutrophils and activated eosinophils, and is attached to the plasma membrane by a GPI-link, thus being truncated and not directly associated with signaling chains. Therefore, CD16b is thought to function in cooperation with other Fc receptors. No homologues for human CD16b have been described in other species. Because of the major role that CD16 plays in the immune response, we have identified and characterized nonhuman primate CD16 homologues. Identification of these homologues is especially important in view of the increasing use of nonhuman primate models to test therapeutic antibodies as well as strategies to control xenograft rejection. We profiled peripheral blood leukocytes for CD16 expression by flow cytometry with a cross-reactive anti-human CD16 monoclonal antibody (Figure 6.1). Traditionally, CD16 has been used to phenotype nonhuman primate natural killer cells with cross-reactive anti-human CD16 antibodies. However, there are no published reports on the expression of these molecules on nonhuman primate granulocytes. CD16 is undetectable on the cell surface of rhesus macaques, cynomolgus macaques and baboons, although it is expressed

on natural killer cells and some monocytes. Surprisingly, CD16 was detected on sooty mangabey neutrophils as well as natural killer cells and a few monocytes from this species. Genetic analysis of CD16 mRNA in nonhuman primates confirms the absence of CD16b in rhesus macaques, cynomolgus macaques, and baboons. Indeed, all the transcripts isolated from these three species appear to be more related to CD16a. The same was true of transcripts isolated from sooty mangabey. In disagreement with results obtained by flow cytometry in sooty mangabeys, genetic analysis shows only presence of CD16a related transcripts in this species. Sooty mangabey CD16 transcripts differ only at the genetic level from the corresponding baboon transcripts. Indeed, the deduced CD16 amino acid sequences are identical in sooty mangabeys and baboons. Macaque CD16 deduced amino acid sequences are also highly conserved with baboon/mangabey CD16 sequences. The differences in the expression profile of sooty mangabey CD16 when compared to other nonhuman primate species could be explained on the basis of a differential regulation of the expression of the identified sooty mangabey CD16 gene, which in turn would be the result of differences in promoter elements responsible for restrictive tissue type expression. Alternatively, as demonstrated in humans, sooty mangabeys may possess a second CD16 gene.

To assess IgG/nonhuman primate CD16 interactions, we generated a high expression recombinant sooty mangabey CD16 HeLa cell clone. The resolved crystal structure of a human CD16/IgG1 Fc complex and mutational studies have determined important residues of interaction on both molecules. The majority of residues that in humans are responsible for Fc interaction are conserved in nonhuman primate CD16

molecules. A few human-nonhuman primate substitutions are conserved in all four nonhuman primate species. Therefore, we could predict that the binding properties of nonhuman primate CD16 molecules are similar to those of human CD16. Indeed, our results show that recombinant mangabey CD16 is capable of binding heat-aggregated polyclonal IgG from rhesus macaques, cynomolgus macaques, baboons and sooty mangabeys. The recombinant CD16 molecule also binds heat-aggregated human IgG1 and IgG2 (Figure 6.1). However, this molecule does not appreciably bind the other human antibody classes. These results indicate that the subclass interaction properties of sooty mangabey/baboon CD16 (and probably macaque CD16) differ from the corresponding human properties. Such a finding has important implications for the testing of therapeutic antibodies in these species. IgG2 therapeutics tested in these animals are likely to be able to activate cells, ex. NK cells, which would not be activated in humans. On the other hand, interactions with IgG1 molecules would be different for human and nonhuman primate neutrophils, at least for the three species in which CD16 is not expressed in neutrophils. While efficacy studies of therapeutic antibodies ultimately must be carried out in humans, trials in nonhuman primate species are especially important for evaluation of safety. Without considering the differences existing between human and nonhuman primate CD16, there is a potential to overestimate or underestimate health risks and therapeutic values of monoclonal antibodies or other molecules containing antibody Fc regions.

Use of nonhuman primates in biomedical research is based on the premise that these species are physiologically similar to humans. As biomedical research becomes

more technical and more dependent on refined molecular targeting, it has necessary to clearly delineate the distinctions of these species from humans. Our characterization of nonhuman primate immunoglobulins and Fc receptors shows presence of conserved traits, e.g. IgD structure and CD89/ligand interaction, and non-conserved traits, e.g. aspects of CD16 expression and CD16/ligand interactions. The study of the immune system and immune responses in nonhuman primates is likely to lead to new insight into human biology and human diseases.

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Appendix A

Supplemental Information Related to the Identification of IgD in Nonhuman Primates and Dogs: Additional Considerations

Because of the advent of the various genome projects and increasing availability of genetic information through GenBank and other community databases, it has become possible to access large amounts of genetic information. The Human Genome Project was officially launched in 1990 and completed in April 2003 when two simultaneous reports on the completion of the human genome sequencing were released in *Science* and *Nature*. Sequencing of genomes of other species, including several mammals, is in various stages of completion. 2005 marks the official release of the complete chimpanzee genome, and the dog genome is also soon to be released. The information obtained through the Human Genome Project is expected to further our understanding of diseases, thus leading to new cures. Just as the Human Genome Project is expected to advance human medicine, the Dog Genome Project is expected to advance veterinarian medicine. Considering the intimate relationship existing between the immune system and disease, it is then reasonable to exploit the information available from the various genome projects to understand the biological role of IgD. Therefore, in the next few pages, we will use sequence information from different genome projects to answer biological questions related to IgD genes.

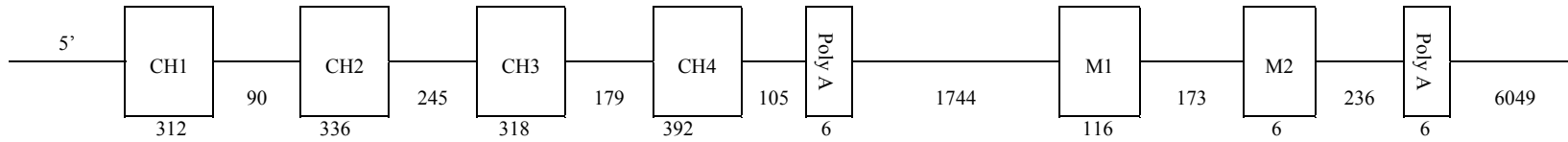
Is the chimpanzee immunoglobulin heavy chain (IGH) locus similar to the human locus?

What can we learn about the evolution of human immunoglobulins?

Upon identifying a contig from GenBank that encodes the chimpanzee *IGHD* (accession number NW_115908), we examined the contig for flanking genes and the exon/intron arrangement of *IGHD*. This revealed that chimpanzee *IGHD* is flanked by *IGHM* and *IGHG3* (the genes encoding the immunoglobulin heavy chains of IgM and IgG3) on the contig. This is the same gene arrangement as is found in the human immunoglobulin heavy chain locus.

As it is characteristic of all described *IGHD* genes, the *IGHM* gene is found upstream of the *IGHD* gene (Figure A.1). The intergenic space between chimpanzee *IGHM* and *IGHD* is 6.195kb, slightly larger than the intergenic distance of the human orthologues (6.049 kb based on GenBank contig NC_00014). Comparing the predicted *IGHM* transcript with sequences available through GenBank, we found a Gnomon predicted mRNA (accession number XM_522973) constructed from the same contig. Such a Gnomon agrees with our prediction, except that it contains additional nucleotides 5' of the expected CH1 exon boundary. The chimpanzee *IGHM* gene is structured as the human gene, with four exons encoding the CH1-CH4 domains followed by two exons encoding the transmembrane domain and the cytoplasmic tail. Figure A.2 shows the alignment of the deduced amino acid sequence of chimpanzee *IGHM* with that of two human *IGHM* alleles (*IGHM*01* and *IGHM*03*). As expected, all cysteines involved in disulfide bonds within the immunoglobulin domains and linking the four chains of IgM are preserved in the chimpanzee. Five N-glycosylation sites are present at positions identical to those found in the human IgM heavy chain. A few amino

Human Chromosome 14q32.33 (GenBank accession NC_000014)



Chimpanzee Chromosome 14 (GenBank accession NW_115908)

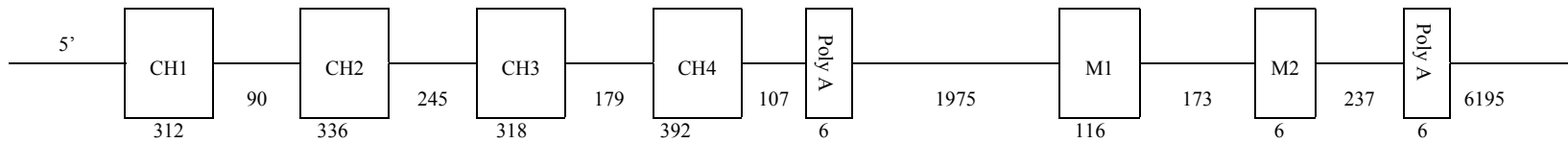


Figure A.1. Chain diagram of human and chimpanzee *IGHM* genes . Numbers indicate nucleotides in exons or introns.

CH1

Human*01 GSASAPTLFPLV**S**CENSPSDTSSVAVG**CLA**QDFLPDSITLSWYK**K***NNS*DISSTRGFPSVL
Human*03 GSASAPTLFPLV**S**CENSPSDTSSVAVG**CLA**QDFLPDSITLSWYK**K***NNS*DISSTRGFPSVL
Chimpanzee GSASAPTLFPLV**S**CENSPSDTSSVAVG**CLA**QDFLPDSITLSWYK**K***NNS*DISSTRGFPSVL

▼ CH2

Human*01 RGGKYAATSQVLLPSKDV**M**QGTDEHV**V**CKVQH**P**NGNKEKNVLPV**I**AELPPKVS**V**FVPPR
Human*03 RGGKYAATSQVLLPSKDV**M**QGTDEHV**V**CKVQH**P**NGNKEKNVLPV**I**AELPPKVS**V**FVPPR
Chimpanzee RGGKYAATSQVLLPSKEV**M**QGTDEHV**V**CKVQH**P**NGNKEKNVLPV**T**AELPPKVS**I**FVPPR

Human*01 DGFFGNPR**K**SKLI**C**QATGFSPRQIQVSWLREGKQ**V**GS**G**VTTDQVQAEAK**E**SGPTTYKVTS
Human*03 DGFFGNPR**K**SKLI**C**QATGFSPRQIQVSWLREGKQ**V**GS**G**VTTDQVQAEAK**E**SGPTTYKVTS
Chimpanzee DGFFGNPR**S**SKLI**C**QATGFSPRQIQVSWLREGKQ**V**GS**G**VTTDQVQAEAK**Q**SGPTTYKVTS

▼ CH3

Human*01 TLTIKESDWL**G**Q**S**MFTCRVDHRGLTFQ**Q***NAS*SM**C**VPDQDTAIRVFAIPPSFASIFLTKST
Human*03 TLTIKESDWL**S**Q**S**MFTCRVDHRGLTFQ**Q***NAS*SM**C**VPDQDTAIRVFAIPPSFASIFLTKST
Chimpanzee TLTIKESDWL**S**Q**S**VFTCRVDHRGLTFQ**Q***NAS*SM**C**SPGFDTAIRVFAIPPSFASIFLTKST

Human*01 KLT**C**LVTDLTTYDS**V**TISWTRQNGEAVK**T**HT**N**ISESH**P***NAT*FSAVGEASICE**D**DDWNSGER
Human*03 KLT**C**LVTDLTTYDS**V**TISWTRQNGEAVK**T**HT**N**ISESH**P***NAT*FSAVGEASICE**D**DDWNSGER
Chimpanzee KLT**A**LVTDLTTYDS**L**TISWTRQNGEAVK**T**HT**N**ISESH**P***NAT*FSAVGEASICE**D**DDWNSGER

▼ CH4

Human*01 FT**C**TVTHTDLPSPLKQ**T**ISRPK**G**VALHRPDVYLLPPAREQLNLRES**A**TIT**C**LV**T**GFSPAD
Human*03 FT**C**TVTHTDLPSPLKQ**T**ISRPK**G**VALHRPDVYLLPPAREQLNLRES**A**TIT**C**LV**T**GFSPAD
Chimpanzee FT**C**TVTHTDLPSPLKQ**T**ISRPK**E**VALHRPDVYLLPPAREQLNLRE**L**A**T**IT**C**LV**T**GFSPAD

Human*01 VFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETY**T****C**V-AHEAL
Human*03 VFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETY**T****C**VVAHEAL
Chimpanzee VFVQWMQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETY**T****C**VVAHEAL

Human*01 PNRVTERTVDKSTGKPTLY**N**VSLVMSDTAG**T****C****Y**
Human*03 PNRVTERTVDKSTGKPTLY**N**VSLVMSDTAG**T****C****Y**
Chimpanzee PNRVTERTVDKSTGKPTLY**N**VSLVMSDTAG**T****C****Y**

Tail of Membrane bound IgM

M1 ▼M2

Human GEVSADEEGFENLWATASTFIVL**F**LLSLFYSTTVTL**F**KVK
Chimpanzee GEVSADEEGFENLWATASTFIVL**F**LLSLFYSTTVTL**F**KVK

Figure A.2. Alignment of chimpanzee and human IgM heavy chains. The chimpanzee (Chimp) *IGHM* deduced amino acid sequence were obtained through analysis of a contig on chromosome 14 (GenBank accession number NW_115908) and compared with two human alleles *IGHM*01* (GenBank X14940) and *IGHM*03* (GenBank X57331). Chimpanzee *IGHM* is most like human allele *IGHM*03*, indicating that this allele maybe more ancestral. The tail of the membrane bound form of IgM is identical for all the sequences. Amino acid differences are underlined. Arrowheads indicate the start of each domain encoded by separate exons. Potential N-glycosylation sites and cysteines involved in disulfide bonds are italicized and bolded. Dashes indicate deleted residues.

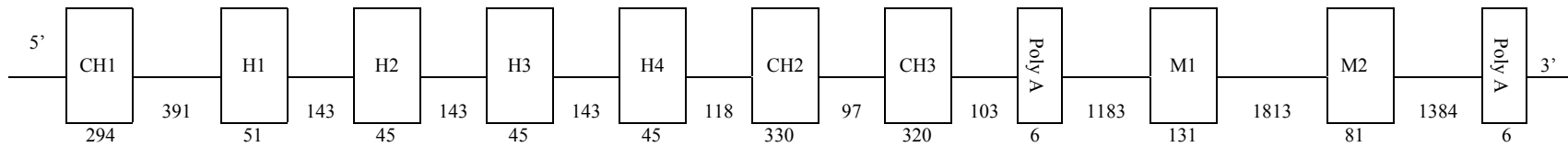
acid substitutions are found in each of the CH domains, but no substitutions are located in the secretory tail, the transmembrane domain and cytoplasmic tail.

The chimpanzee sequence is more conserved with *IGHM*03* than with *IGHM*01*. The chimpanzee *IGHM* shares 96.0% amino acid identity with *IGHM*01* and 97.1% identity with *IGHM*03*. Chimpanzee *IGHM* has a phenylalanine at CH1 position 40, serine at CH2 position 87 and a valine at CH4 position 93 as is the case for human *IGHM*03*, whereas human *IGHM*01* has a leucine at the first position, a glycine at the second position and a deletion at the latter position. This finding suggests the possibility that the human allele *IGHM*03* may represent a more ancestral allele, whereas *IGHM*01* may represent a derived allele. Therefore, we examined such a possibility. At the nucleotide level, the chimpanzee identities with the respective human alleles *IGHM*01* and *IGHM*03* are 97.4% and 98.1%, respectively. There are 30 nucleotide substitutions in chimpanzee *IGHM* when compared to human *IGHM*. Of these substitutions, 26 are different from both human alleles and the remaining 4 are only different from *IGHM*01*. It is possible that these substitutions may have arisen from two mutation events, once in the chimpanzee gene and once in *IGHM*01*, but this is highly unlikely for all four positions. The Chimpanzee Sequencing and Analysis Consortium (2005) has reported that the error rate for assignment of human single nucleotide polymorphisms (SNPs) as ancestral (on the basis of their presence in the chimpanzee sequence) is estimated at ~1.6% overall and 12% when the chimpanzee sequence occurs at a TG and human polymorphisms are CG and TG. Indeed, CpG dinucleotides are hotspots for deamination of C to yield T (Hwang and Green, 2004). Thirty % of the chimpanzee *IGHM* substitutions are at a CG/TG dinucleotides compared to the 12.5% that

would be expected by unbiased random mutation of the gene. Only one of the four chimpanzee substitutions differing from a single human allele is a CG/TG substitution (this substitution is TG in the chimpanzee sequence and *IGHM*03*). Together, these data suggest that the human allele *IGHM*03* may be ancestral. Further analysis of other primate IGHM sequences is required to determine whether *IGHM*01* is likely derived or possibly originates from an ancestral polymorphism.

A putative chimpanzee *IGHG3* was found ~90kb downstream of *IGHD* (Figure A.3). This intergenic distance is larger than that found in humans, (65 kb between *IGHD* and *IGHG3*). Due to the incomplete resolution of the contig sequence, the center portion of the *IGHG3* sequence encoding part of the hinge region, CH2 and the 5' end of CH3 could not be examined (Figure A.3 and A.4). The last exon encoding the second half of the transmembrane domain and cytoplasmic tail could not be identified. It is likely that the contig sequence terminates prior to this portion of the chimpanzee *IGHG3*. Despite the incomplete sequence, it is clear that the gene, if functional, would be classified as *IGHG3*. Indeed, two complete hinge exons and a portion of a third exon encoding amino acids identical to those of the human IgG3 heavy chain can be identified. In humans, the IgG hinge region is the most defining characteristic of the IgG subclasses, and only the human IgG3 subclass has a hinge encoded by multiple exons, the number of which varies depending on the allele (LeFranc and LeFranc, 2001). Additionally, as is the case for human *IGHG3*, the chimpanzee gene is found downstream of *IGHD*. In the chimpanzee IgG3 CH1, CH3 and available portion of the hinge region, all cysteines involved in inter-

Human Chromosome 14q32.33 (GenBank accession NC_000014)



Chimpanzee Chromosome 14 (GenBank accession NW_1159908)

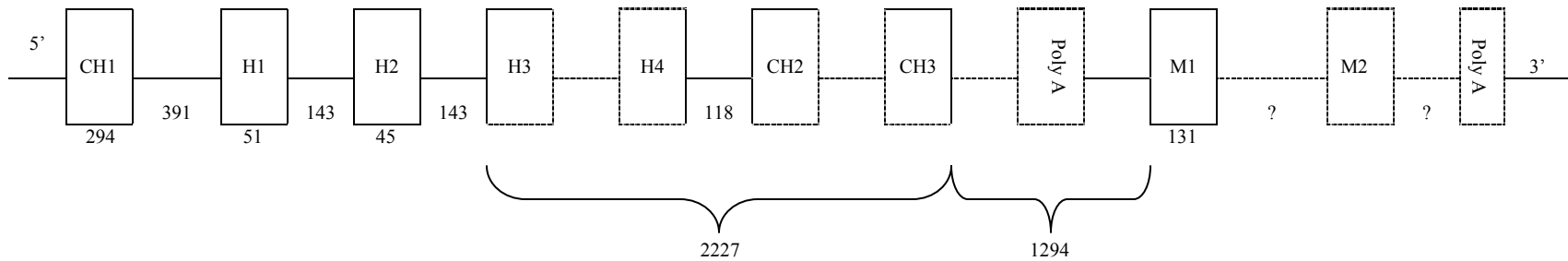


Figure A.3. Chain diagram of human and chimpanzee *IGHG3* genes. Numbers indicate nucleotides in exons or introns.

Domains that have not been identified are indicated by dashed lines.

Figure A.4. (Next Page) Alignment of chimpanzee and human IgG3 heavy chains.

Chimpanzee (Chimp) *IGHG3* derived amino acid sequences were obtained through analysis of GenBank sequences including a contig on chromosome 14 (accession number NW_115908), from which *IGHG3* had not previously been identified, and two partial mRNA sequences denoted as allele A1 (GenBank AF300434) and allele A2 (GenBank AF300435). Human alleles of the IgG3 heavy chain exist with varying numbers of hinge exons (H1, H2, H3 and H4). Although incomplete, the available chimpanzee sequences also vary in number of hinge exons. Therefore multiple IgG3 alleles with varying numbers of hinge exon repeat may represent polymorphisms of a common ancestor of both primate species. Amino acid differences are underlined. Arrowheads indicate the start of each domain encoded by separate exons. Potential N-glycosylation sites and cysteines involved in disulfide bonds are italicized and bolded. Dashes indicate residues absent in the sequences, whereas asterisks indicate unresolved sequences from the contig. Sequences are compared to the human allele *IGHG3*03* (GenBank X03604). The tail of the membrane bound form of IgG3 is composed of exons M1 and M2. Only M1 is shown here since the chimpanzee contig did not extend through to the M2 exon.

and intra- chain disulfide bonds are conserved with human IgG3. The N-glycosylation site CH3 N52 is also conserved in the chimpanzee protein.

Two partial sequences of chimpanzee *IGHG3* have been deposited in GenBank by Fortenfant *et al.* (accession numbers AF300434 and AF300435). These short cDNA sequence fragments (CH1 codon 55 to CH2 codon 31) contain 764nt that overlap with the contig sequence. These sequences are designated in GenBank as alleles A1 (AF300434) and A2 (AF300435) and share 98.0% nucleic acid identity with each other and 97.4% nucleic acid identity with the contig sequence for this overlapping region. Each of these smaller sequences have hinge regions encoded by two hinge exons in contrast to the contig sequence that contains three or more hinge exons (Figure A.4). Thus, chimpanzee *IGHG3* exists in multiple alleles, some of which differ because of the number of hinge exons they possess, as found in humans. These data suggest the possibility that the human *IGHG3* alleles were established before the phylogenetic split of humans and chimpanzees. Alternatively, duplication of the hinge exons may have occurred separately in humans and chimpanzees, despite the short period of time since their divergence.

Overall, the chimpanzee *IGH* locus region surrounding *IGHD* is quite similar to the human *IGH* locus. The intron sizes in all three genes are quite similar to those found in humans (Figure 4.1, A.1, A.3). Where there are differences in intron sizes, the majority of differences are due to longer chimpanzee introns. The locus region examined is much larger in the chimpanzee primarily as a result of longer intergenic distances. All identified chimpanzee introns for *IGHG3*, *IGHD* and *IGHM* have canonical GT...AG boundaries.

Future analysis of other immunoglobulin genes in chimpanzees and other hominoid species would likely help elucidate the extent to which inheritance from a common primate ancestor contributed to human immunoglobulin polymorphisms. Here, results from the analysis of chimpanzee *IGHM* and *IGHG3* indicated that more than one allele of many human genes are ancestral. Such finding would support the view that there was a diverse genetic pool among the founders of our species.

Humans have two alleles of IGHD, is one more ancestral than the other?

The two described alleles of human *IGHD* (*IGHD*01* and *IGHD*02*) differ by two silent nucleotide substitutions, one at H1 codon 9 position 3 (C versus A) and one at CH3 codon 9 position 3 (T versus C). Both of these nucleotides are conserved in chimpanzees, baboons, sooty mangabeys and macaques with the *IGHD*02*, indicating that *IGHD*01* is more ancestral.

What does our analysis tell us about the limitations of the bioinformatics program Gnomon to predict open reading frames from the eukaryotic genome projects?

The bioinformatics program Gnomon is used to predict gene open reading frames and create putative mRNA from DNA sequences. Through the release of different genomes from the various genome projects, many of these putative mRNAs have been deposited into GenBank. As mentioned above, a chimpanzee *IGHM* mRNA was constructed in this manner, but the predicted transcript mistakenly incorporated sequence 5' to the proper start of the gene. This may represent a limitation of Gnomon. Immunoglobulin heavy region constant

genes lack a canonical start codon and promoter, because their transcription begins at the immunoglobulin genes encoding the variable domain, which is created through somatic recombination. Therefore Gnomon may inappropriately identify start sequences for the immunoglobulin constant heavy genes located upstream of their real start sequences.

By screening GenBank, we also found Gnomon predicted IgD-like mRNAs for chimpanzee (GenBank XM_51026) and for dog (GenBank XP_548647). Both of these sequences were assembled incorrectly and illustrate potential pitfalls of Gnomon analysis. The chimpanzee mRNA and the deduced protein sequences are highly conserved with those of human *IGHD* CH2 and CH3 exons and IgD respectively. However, other exons of the *IGHD* gene are not present. 5' of the CH2 exon the putative mRNA is conserved with CH1-CH2 intron sequences and 3' of the CH3 exon sequence is taken from *IGHG3* found downstream of *IGHD*. Additionally, the CH2 exon sequence has a single nucleotide insertion that shifts the reading frame. The dog sequence has similar mistakes made in its assembly, with the CH2 exon being preceded by sequences derived from the H2-CH2 intron. Mapping out these genes may provide possible explanations for these errors.

Analysis of the chimpanzee contig for *IGHD* exons homologous to human *IGHD* exon reveals seven of the eight anticipated exons (Figure 4.1). The putative second exon encoding the tail of the transmembrane bound IgD is missing. This second exon has been found in all the other characterized mammalian *IGHD* genes and encodes two amino acids and the stop codon. Presumably, this second exon would be present in a region of the contig where the sequence has not yet been resolved (consistent with the hypothesis that the intron between the first and second transmembrane encoding exons is of similar size to that present

in humans). Indeed, the intron sizes of the remaining *IGHD* gene are very close to those of their corresponding human counterparts. Therefore, Gnomon may have inappropriately incorporated a part of *IGHG3* in its predicted chimpanzee IgD-like mRNA, because the appropriate stop codon was not found as a result of the incomplete resolution of the contig it used. The incorporation of an intron at the 5' end of the predicted mRNA may be explained because of the lack of any stop codons in this sequence. This is also true for the predicted dog sequence, where the problem of predicting the correct reading frame may be compounded by the H1-CH3 intron having a cryptic 5' end rather than the canonical GT dinucleotide.

Gnomon is a useful tool for identifying genes. However, it appears prone to errors. These errors may be due to incomplete sequence resolution, presence of unusual introns, and presence of non-classical genes. The use of this program may lead to underestimate numbers of genes as a result of predictions deriving from a combination of two actual genes, as shown for chimpanzee *IGHD* and *IGHG3*. Therefore, Gnomon is a practical tool for an initial genome screening. However, results related to predicted genes should be carefully evaluated.

Do the IGHG of carnivora members use a cryptic splice site for the H2-CH2 intron?

As determined by comparing mRNA with the DNA sequence, dog *IGHD* has two hinge region encoding exons. Examination of the DNA sequence alone does not readily reveal the second exon (H2), since the contig sequence does not have the canonical GT at the start of the intron between H2 and CH2 (Figure A.5). One simple explanation is that

A.

```

Dog   catctcccttttcctccctag/TGCCTCCCACCAGCCACACC
Panda cacgccccttttcccccgcag/TGCCCTCCACCAGCCACACC

Dog   CAGACGCAAGCCCAGGAGCCAGGATGCCCAGTGGACACCA
Panda CAGACACAAGCCGCGGAGCCAGGAAGCCCAGGGGATGCCA

Dog   TCCTCAGAG/gccagttcct-ggggtgcaga
Panda AGCACAGAG/gccagttccttggggcacaga

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B.

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Dog   VPPTSHTQTQAQEPGCPVDILR
Panda VPSTSHTQTQAAEPGSPGDAKHR

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Figure A.5. Dog and putative panda IgD second hinge region exons. Alignments of the dog and panda *IGHD* H2 exon gene sequences with nucleotides of the flanking 5' and 3' introns (A) and the deduce amino acid sequences (B) are shown. The dog *IGHD* exon boundaries have been confirmed from mRNA sequence. The 3' exon does not start with the typical GT dinucleotide. The sequence at this exon/intron junction is highly conserved with putative panda H2 3' exon. This suggests that the use of a cryptic splice junction for the 3' end of *IGHD* H2 may be a common feature of carnivora. Panda nucleotides and amino acids different from those of dog are underlined.

the contig sequence contains a miscalled nucleotide at the start of the intron.

Alternatively, the intron/exon boundary may be cryptic. An unpublished DNA sequence of *IGHD* from panda, also a member of carnivora, is available in GenBank (AY818394). The authors of the panda submission only identify the first hinge exon, but when we screened the gene for a possible second exon we found a sequence with 66% nucleotide identity and 69.6% deduced amino acid sequence identity with the second exon of dog *IGHD* (Figure A5). The intron that would be present 5' of the putative panda H2 exon follows the canonical GT...AG rule, but the 3' intron, similarly to what found in dogs, does not start with GT. In panda *IGHD*, the putative H2 preceding the predicted exon/intron junction and the first ten nucleotides of the intron are identical to the similar dog sequence. Sequencing of panda *IGHD* mRNA could easily determine whether or not this is a true H2. Assuming that the genomic sequences are correct and that the 5' end of the introns are correctly identified, then the intron would follow a GC...AG splice type. Less than 1% of introns are spliced by the U2 spliceosome that contain a motif GC...AG (Burset *et al.*, 2001). However, these introns have 5' sequences that are strongly conserved with the complement sequence of the U1 snRNA (GAG/gcaagt) (Abril *et al.*, 2005). This sequence is only one nucleotide different from the sequences at the 5' end of the H2-CH2 introns of dog and panda *IGHD* genes. Taken together, the information from dog and panda sequences suggests that an *IGHD* of carnivora members may possess H2 exons with cryptic 3' exon/intron boundaries.

How do the multiple immunoglobulin domains of fish IgD correspond to the mammalian IgD domains?

Fish IgD is structurally quite different from mammalian IgD. Fish IgD heavy chains have no hinge, require splicing of IgM CH1 to the N-terminus to provide the cysteines necessary to form disulfide bonds with the immunoglobulin light chains, and consist of up to seven immunoglobulin domains, which may be repeated depending on the species (Wilson *et al.*, 1997; Stenvik and Jørgensen, 2000; Hirono *et al.*, 2003; Srisapoome *et al.*, 2004; Savan *et al.*, 2005; Saha *et al.*, 2004; Hordvik, 2002; Danilova *et al.*, 2005; Bengtén *et al.*, 2002; Hansen *et al.*, 2005; Hordvik *et al.*, 1999). These structural differences have made it difficult to immediately correlate the different immunoglobulin domains of teleost fish to those in mammals. A general consensus present in the literature is that the teleost CH1 and mammalian CH1, as well as teleost CH5 and mammalian CH2 domains, correspond most closely to each other. On the other hand, there is disagreement about whether teleost CH6 or CH7, most closely correspond to mammal CH3 (Stenvik and Jørgensen, 2000; Hordvik *et al.*, 1999). To test the relationship of mammalian IgD CH3 to fish IgD CH6 and CH7 we constructed phylograms using the different mammal CH3 and teleost CH1, CH5 (or Atlantic cod CH2), CH6 and CH7 (Figure A.6). The resulting phylogenetic tree matches the accepted taxonomy of mammals, with the exception that the relationship between Old World monkeys is not resolved (probably because of the shallow differences within the group). Fish CH6 appears to be the domain most homologous domain to mammalian CH3. Accordingly, both of their clades share a common node that follows the split of the teleost CH6 and CH7 clades.

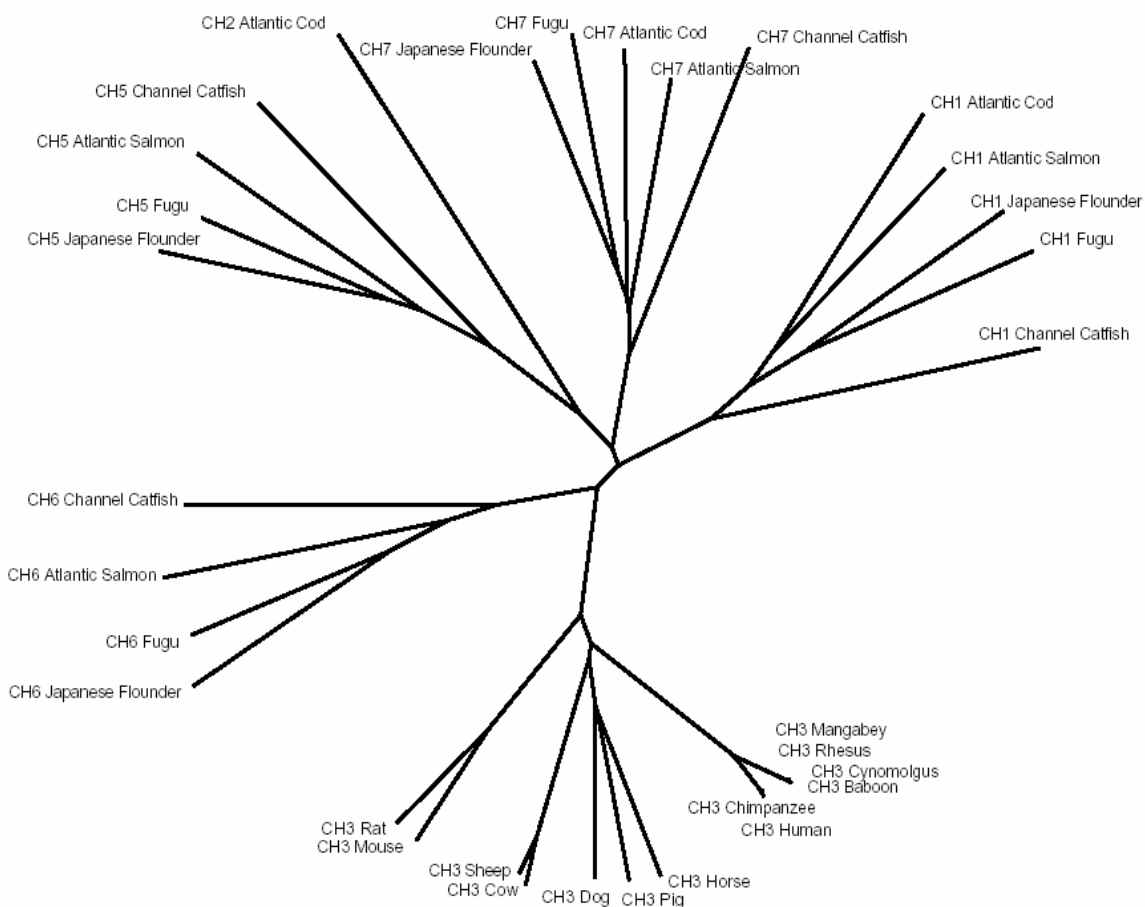


Figure A.6. Phylogenetic relationships of mammalian IgD CH3 domains and fish IgD CH domains. Neighbor joining tree of IgD was constructed with deduced amino acid sequences using the CLUSTAL method. The CH6 domains of fish cluster most closely with mammalian CH3, indicating that these domains are more homologous than fish CH7 domains and mammalian CH3. CH3 differences in primates are small and resolved into only two clades. GenBank accession numbers for mammals are included in the Materials and Methods section of Chapter 4. GenBank accession numbers for fish are: channel catfish U67437, fugu AB159482, Atlantic salmon AF141605, Japanese flounder AB052658, Atlantic cod AF 155199 and halibut AY077849.

What is the origin of the IgD secretory tail and is secretory IgD a common feature of species with IgD?

Of the immunoglobulin heavy chain genes, only *IGHD* has a separate exon that encodes the hydrophilic secretory tail (Lefranc and Lefrance, 2001). This tail has been identified in some of the species possessing a *IGHD* gene and it is highly diverse among these species (Figure A.7) (White *et al.*, 1985; Mushinski *et al.*, 1980; Zhao and Hammarström, 2003; Zhao *et al.*, 2002; Wagner *et al.*, 2004, Bengtén *et al.*, 2002). In channel catfish the IgD membrane bound and secreted forms are encoded by separate genes (Bengtén *et al.*, 2002). One secretory tail has been confirmed for mouse IgD. A second putative tail has also been identified and its presence confirmed by Northern blot analysis (Cheng *et al.*, 1982). The chimpanzee CH-S is the least conserved of the *IGHD* exons when compared to human *IGHD*. Although a polyadenylation site is found 5' to the first dog *IGHD* transmembrane domain exon, we could not identify a CH-S with homology to CH-S from other species. Mouse CH-S is much longer than human CH-S and it only shares identity at the nucleotide level with the carboxy-terminus of human CH-S (White *et al.*, 1985). In fish, CH-S has only been described for some species. In channel catfish, two separate *IGHD* genes encode secretory and membrane bound IgD (Bengtén *et al.*, 2002). It is possible that similarly to the hinge exon, the secretory tail has evolved many times. For several species it has not yet been established whether or not IgD is secreted. Therefore, the importance of secreted IgD in the vertebrate immune responses cannot be fully evaluated.

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Human          -----Y-VTDH---GPMK
Chimpanzee    -----Y-VTDR---GPVK
Rat           GCY---HLLPESDGPPRRPDGPAFP
Mouse (Putative) GCY---HLLPESDGPSRRPDGPALA
Mouse         EDFLFKIYSKSYKISAR---TSHKA
Horse         GPSHGSSSGSRAGQPQE---TSSHA
Pig           PLLIQQRLGAEWKASKRAPASPE-A

```

Figure A.7. Alignments of characterized IgD secretory tails from mammals. The poor conservation of the IgD secretory tails may be due to the exon evolving multiple times. It has been hypothesized that mice may use more than one IgD secretory tail (Cheng *et al.*, 1982); the unconfirmed mouse IgD tail is shown here as putative (both tails are from GenBank accession number J00450).

Appendix B

IgG Subclass Evolution and Its Implications for IgG Fc Receptor Interactions

How do human and nonhuman primate IgG subclasses relate to each other? What do these relationships tell us about their potential functional properties including interactions with IgG Fc receptors?

IgG subclasses are often determined for immunological studies in mice and humans, as they are a major indicator of different types of immune responses (Jefferis *et al.*, 1994). For example, human natural killer cell ADCC responses are associated with IgG1 and IgG3, because CD16 binds these subclasses (Tamm and Schmidt, 1997). It is desirable to similarly measure different IgG subclass responses in nonhuman primates (Shearr *et al.*, 1999). However, reagents that can be used for this purpose are not currently available. In addition, it is unknown whether or not the four nonhuman primate IgG subclasses correlate functionally with their human counterparts. In particular, it is not known whether or not nonhuman primate IgG subclasses are able to engage immune effector functions through CD16. If the IgG subclasses of human and nonhuman primates were inherited from a common ancestor, then it would be reasonable to hypothesize that they share cognate effector functions.

Phylogenetic analysis of immunoglobulin gamma heavy chains from different species reveals that multiple IgG subclasses likely arose repeatedly from a single ancestral gene in multiple species (Figure B.1; Scinicariello *et al.*, in press; Wagner *et al.*, 2002; Flanagan and Rabbits, 1982). For primates, this analysis produces two separate IgG

clades, one for hominoids and one for old world monkeys. Following branching of these two clades, the IgG heavy chains cluster together by subclasses. Therefore, IgG heavy chain gene duplication appears to have happened separately in Old World monkeys and in hominoids, but prior to radiation of the representative species from each group. If this analysis represents the true evolutionary history of these subclasses, then there is no *a priori* reason for predicting that the IgG subclasses of Old World primates have functions in common with the human IgG subclasses. By contrast, IgG subclasses from other hominoids appear to be true orthologues of human subclasses. Phylogenetic analysis of IgG genes should take into account that gene conversion is likely to have shaped these genes, as shown for human *IGHG* genes (Lefranc *et al.*, 1986). Such genetic events would lead to overestimating how closely related these genes are in Old World monkeys.

Old World monkey IgG subclasses are similar to those of humans. In humans, baboons, sooty mangabeys and macaques (rhesus, cynomolgus, and pig-tailed), the lower hinge of IgG2 molecules has a deletion of one amino acid. In addition in baboons and humans, there is a common substitution of a glycine with an alanine in the IgG2 lower hinge region. Phylogenetic analysis indicates these similarities represent convergent evolution (Figure B.1). Human subclass differences in the IgG lower hinge regions account for their varying capacities to bind to IgG Fc receptors, including CD16 (Tamm and Schimdt, 1997). If nonhuman primate IgG subclass differences alter the ability to form IgG/Fc receptor interactions, then convergent evolution would help to substantiate the hypothesis of the selective advantage provided by IgG subclasses that vary in their capacity to initiate effector functions. Homologues of CD16 have been identified in other

Figure B.1. (Page 253) Phylogenetic relationships of IgG heavy chains from different species. The neighbor joining (NJ) tree was constructed from the amino acid sequences of the immunoglobulin gamma heavy chains of various mammalian species, two birds and an amphibian. The NJ tree indicates that multiple IgG subclasses emerged from one progenitor IgG in many groups after splits from other groups. The four subclasses of old world monkeys appear to have evolved separately from those of hominoids after the two groups split. Bootstrapping of the NJ tree was performed with 10,000 replicates. The GenBank accession numbers for the reported species are: Australian brushtail possum (Possum) (AF157619); baboon (Paca) *IGHG1* (AY125048), *IGHG2* (AY125049), *IGHG3* (AY125050), and *IGHG4* (AY125051); camel *IGHG1* (CDIGGC); cat *IGHG1a* (AB016710) and *IGHG1b* (AB016711); chicken (X07174); chimpanzee *IGHG1* (X65284-5, X61310-1); cow *IGHG1* (X16701), *IGHG2* (X16702), *IGHG3a* (BTU63638) and *IGHG3b* (BTU63639); dog *IGHG a* (AF354264), *IGHG b* (AF354265), *IGHG c* (AF354266), and *IGHG d* (AF354267); duck (X65219); echidna (AF416949); gray short-tailed opossum (Opossum) *IGHG* (AF035195); hamster IgG2 (U17166); horse *IGHG1* (AJ302055), *IGHG2* (AJ302056), *IGHG3* (AJ312379), *IGHG4* (AJ302057), *IGHG5* (AJ312380), *IGHG6* (AJ312381), IgG7 (AY445517); human (Hm) *IGHG1* (J00228), *IGHG2* (J00230), *IGHG3* (D78345), and *IGHG4* (K01316); mink (L07788); mouse *IGHG1* (D78344), *IGHG2a* (V00825), *IGHG2b* (J00461), and *IGHG3* (D78343); platypus IgG1 (AY055781), IgG2 (AY055782); rabbit *IGHG* (K00752); rat *IGHG1* (M28670), *IGHG2a* (M28669), *IGHG2b* (M28671), *IGHG2c* (X07189); rhesus macaque (Mamu) *IGHG1* (AY292507), *IGHG2* (AY292519), *IGHG3* (AY292513), and

IGHG4 (AY292521, AY292522, AY292524, AY292525); sheep *IGHG1* (X69797) and *IGHG2* (X70983); sooty mangabey (Ceat) *IGHG1* (AY544376), *IGHG2* (AY544377), *IGHG3* (AY544378), and *IGHG4* (AY544379), pig *IGHG1* (U03778), *IGHG2a* (U03779), *IGHG2b* (U03780) and *IGHG3* (U03781); and *Xenopus* (X15114). IgG sequences for cynomolgus macaque (Mafa) and pig-tailed macaque (Mane) have not yet been published and have not been assigned GenBank accession numbers. The bar indicates the number of substitutions per site.

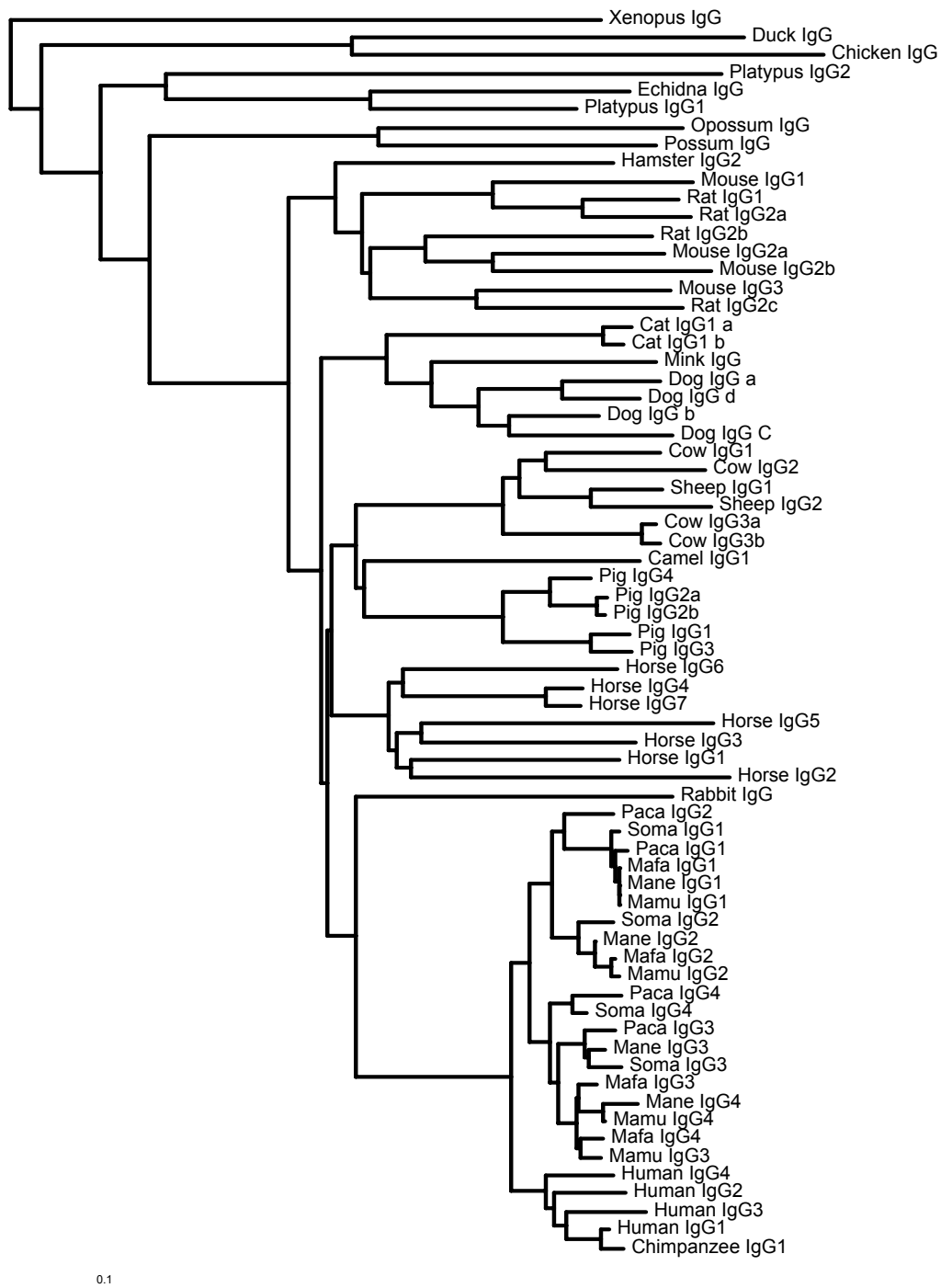


Figure B.1.

other species including mice, cats and cows thus indicating that CD16 predates the development of multiple IgG subclasses in primates (Nishimura *et al.*, 2000; Yan 2000). IgE and IgG are thought to have a common origin as a result of an ancient gene duplication event, which is supported by the ability of mouse IgE to bind CD16 (Arase *et al.*, 2003). Therefore, IgG interactions with CD16 are likely ancient, predating the evolution of the IgG subclasses. Taken together these observations indicate that some subclasses selectively lost the ability to bind CD16, rather than evolving to bind CD16. Under the phylogenetic scenario presented here, Old World monkey IgG subclasses would not inherently be expected to bind or not to bind to CD16 based on relationships to human IgG subclasses.