MECHANISMS OF DOWN-REGULATION OF IMMUNE ACTIVATION AND B-CELL RESPONSES IN THE NATURAL HOSTS OF SIMIAN IMMUNODEFICIENCY VIRUS

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.
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Abbreviations

µM	micromolar
µm	micrometer
AAV	adeno-associated virus
ADCC	antibody-dependent cellular cytotoxicity
AGM	African Green monkey
AIDS	aquired immunodeficiency syndrome
amp	ampicillin
AT2	aldrithiol diphosphate
AZT	Azidothimidine
bp	base pair(s)
BrNAb	broadly neutralising antibody
CD	cluster of differentiation (e.g. CD4)
cDNA	complementary DNA
CDR	complementarity determining region
ct	cycle threshold
CTL	cytotoxic T-lymphocyte
DEPC	diethylpyrocarbonate (diethyl dicarbonate)
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunospot assay
Env	envelope protein (of HIV/SIV)
FACS	fluorescence activated cell scan
FITC	fluorescein isothiocyanate
FKS	fetal calf serum
FoxP3	Forkhead box P3
FR	framework region
g	g-force (9.8m/sec2)
Gag	group-specific antigen (of HIV/SIV)
gag	the gene coding for Gag protein
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
gp	glycoprotein (e.g.gp120)
h	hour
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HCV	hepatitis C virus
HEPS	highly exposed persistent seronegative
HIV	human immunodeficiency virus
IFN	interferon
IFN alpha	interferon alpha
IFN beta	interferon beta
IFN gamma	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-1 alpha	interleukin 1 alpha
IL-1 beta	interleukin 1 beta
IL-10	interleukin 10

IL-12 alpha	interleukin 12 alpha
IL-12 beta	interleukin 12 beta
IL-13	interleukin 13
IL-15	interleukin 15
IL-16	interleukin 16
IL-17	interleukin 17
IL-18	interleukin 18
IL-1Ra	interleukin 1 receptor antagonist
IL-2	interleukin 2
IL-22	interleukin 22
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-7	interleukin 7
IL-8 (CXCL8)	interleukin 8
IP-10 (CXCL10)	interferon gamma-induced protein 10
IRF-7	interferon regulatory factor 7
IRS	immunoregulatory sequence (TLR antagonist)
kb	kilobase(s)
LB	Luria-Bertani
LTNP	long-term nonprogressor
LTR	long terminal repeat
MALT	mucosa-associated lymphoid tissue
MCP-1 (CCL2)	monocyte chemotactic protein 1
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein
MIP-1 alpha	macrophage inflammatory protein 1 alpha
MIP-1 beta	macrophage inflammatory protein 1 beta
ml	milliliter
mM	millimolar
MPER	membrane proximal external region
MyD88	myeloid differentiation primary response gene (88)
NHP	non-human primate
NK cell	natural killer cell
nm	nanometer
OD	optical density
p	protein (e.g. p27)
p.i.	post infection
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed death 1
pDC	plasmacytoid dendritic cell
PDL-1	programmed death ligand 1
PDL-2	programmed death ligand 2
PE	phycoerythrin
Per-CP	peridin chlorophyll
pg	picogram
pVL	plasma virus load
R-848	Resiquimod (TLR7 agonist)
RANTES (CCL5)	Regulated on Activation Normal T Expressed and Secreted
Rh	rhesus
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute

RT	reverse transcriptase
SARS	severe acute respiratory syndrome
sCD40L	soluble CD40 ligand
sec, s	second(s)
SIV	simian immunodeficiency virus
SM	sooty mangabey
SRV	simian retrovirus type D
STLV	simian T-cell lymphotropic virus
Taq	*thermus aquaticus*
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TGF beta	transforming growth factor beta
Th1	T-helper type 1 cell
Th2	T-helper type 2 cell
TLR	Toll-like receptor
Tm	melting temperature
TNF alpha	tumour necrosis factor alpha
Treg	regulatory T-cell
U	unit(s)
VEGF	vascular endothelial growth factor

Zusammenfassung

Natürliche Wirte für das simiane Immundefizienzvirus (SIV), z.B. Afrikanische Grüne Meerkatzen (AGMs) entwickeln trotz hoher Viruslasten kein AIDS. Ebenfalls weisen sie keine detektierbaren Antikörpertiter gegen das virale Gag-Protein auf, das im pathogenen Wirtssytem, z.B. in Rhesusmakaken, immunodominant ist; allerdings entwickeln sie hohe Antikörpertiter gegen das virale Env-Protein. Da SIV-infizierte AGMs im Gegensatz zu Rhesusmakaken keine Ablagerungen von Immunkomplexen in Lymphknoten aufweisen und diese nicht zerstört werden, wurde bisher vermutet, dass die Abwesenheit der anti-Gag-Antikörper für den Schutz gegen AIDS in AGMs eine Rolle spielt, indem sie eine Toleranz gegenüber dem Gag-Protein entwickelt haben, um immunpathologische Auswirkungen der Infektion zu vermeiden. In zwei vorausgegangenen Studien wurde versucht, die vermutete Toleranz des Gag-Proteins zu durchbrechen. Hierfür wurden AGMs mit SIVagmGag-Protein bzw. –DNA immunisiert, was überraschenderweise in beiden Fällen Gag-spezifische Antikörper induzierte und die Hypothese der Toleranz des Gag-Proteins in AGMs widerlegte. Allerdings sanken die anti-Gag-Antikörpertiter nach SIVagm-Infektion auf undetektierbare Niveaus, was eine aktive Unterdrückung der Protein-spezifischen Antikörperantwort in AGMs suggerierte. Um dieses Phänomen zu umgehen war ursprünglich geplant, einen adeno-assoziierten viralen Vektor zu entwickeln, der einen rekombinanten anti-Gag-Antikörper exprimiert. Die mit diesem Vektor „gentherapierten“ AGMs sollten so die gewünschten Antikörper in ihren Muskelzellen produzieren, von wo aus sie unabhängig vom Immunsystem in den Blutkreislauf verteilt würden.

Es wurde jedoch bekannt, dass die humorale Immunantwort gegen Env aufgrund der trimeren Form des Proteins auf der Virusoberfläche T-Zell unabhängig ist, wogegen die humorale Immunantwort gegen Gag-Proteine stark auf T-Zell Hilfe angewiesen ist. Regulatorische Mechanismen, die die Aktivität von T-helfer Zellen unterdrücken, würden daher die Ausbildung einer humoralen anti-Gag- Antwort verhindern, aber keinen Einfluss auf die Induktion von anti-Env-Antikörpern in AGMs haben.

Daher wurde entschieden, einen systematischen Überblick über die Niveaus von immunregulatorischen Biomarkern während der akuten und chronischen Phase der SIV-Infektion zu schaffen, um mögliche herunterregulierende Mechanismen zu identifizieren, die

in einer Abwesenheit von anti-Gag-Antikörpern in natürlichen Wirten für SIV resultieren könnten.

Fortlaufende Plasma- und Zell-Proben, die während der akuten und chronischen Infektionsphase von AGMs und Rhesusmakaken gewonnen wurden, waren aus einer der vorangegangenen Immunisierungsstudien verfügbar. Luminex Multiplex Tests wurden durchgeführt, um die Niveaus von 17 pro- und anti-inflammatorischen Markern in den Plasmaproben zu bestimmen. Hier konnten starke Unterschiede in der Immunaktivierung von AGMs und Rhesusmakaken während der akuten Infektionsphase festgestellt werden, wobei letztere eine wesentlich höhere Immunaktivierung aufwiesen als die AGMs. Zusätzlich wurde ein klarer Unterschied im zeitlichen Auftreten der gemessenen Höchstwerte der Marker festgestellt, denn die Niveaus in den AGMs waren nicht nur niedriger, sondern erhöhten sich zusätzlich später als in den Rhesusmakaken. Dieser zeitliche Unterschied könnte einen wichtigen Faktor für die Entwicklung einer Immuntoleranz gegenüber SIV in AGMs darstellen.

Des Weiteren wurden Real-time PCR Tests für die Detektion von Expressionsniveaus von 32 verschiedenen Biomarkern in PBMCs von AGMs und Rhesusmakaken entwickelt, die nun nützliche Werkzeuge für die Charakterisierung von Immunaktivierungsprofilen auf mRNA Niveau bereitstellen. Um diese Tests zu generieren, mussten zunächst die für die Biomarker kodierenden mRNAs der AGMs sequenziert werden.

Es wurde vermutet, dass Polymorphismen im IRF-7 Gen, das für ein in den Toll-like-Rezeptor 7 (TLR7) Signalweg involviertes Molekül kodiert, für niedrigere Interferon (IFN) alpha-Ausschüttung (und daher eine niedrigere Immunaktivierung) in plasmazytoiden dendritischen Zellen (pDCs) von Rauchmangaben (einer anderen natürlichen Wirtsspezies für SIV) im Vergleich zu Rhesusmakaken verantwortlich sind. Die erhaltene AGM IRF-7 Sequenz wurde mit der von Rauchmangaben, Menschen und Rhesusmakaken verglichen und ein Polymorphismus wurde identifiziert, der nur in den natürlichen Wirten für SIV, AGMs und Rauchmangaben, aber nicht in den heterologen Wirten für SIV, Rhesusmakaken und Menschen, vorhanden ist. Zusätzlich wurde gezeigt, dass AGM pDCs nach Stimulation mit HIV oder SIV wie Rauchmangaben ebenfalls geringere Mengen IFN alpha als pDCs von Rhesusmakaken sezernieren.

Des Weiteren wurde gezeigt, dass AGMs nach der SIV-infektion einen erniedrigten gesamt-Antikörpertiter im Plasma aufweisen, während dieser in Rhesusmakaken zu einer Zunahme tendiert.

Schlussendlich konnten zwei Kandidaten, die für die Abschwächung der T-Helfer Zell-Aktivierung in SIV-infizierten AGMs eine Rolle spielen könnten, identifiziert werden: Eine PD1-PDL1-induzierte Anergie von T-Helfer Zellen sowie die stark verringerte Kapazität der nativen Aktivierung von Gedächtnis-B-Zellen durch eine Aktivierung von TLR7, die aus den reduzierten IFN alpha Ausschüttungen der AGM pDCs nach SIV-Stimulation resultieren.

Es ist bisher nicht bekannt, ob das Phänomen der abwesenden anti-Gag-Antikörper und die Reduktion der gesamt-Antikörpertiter nach SIV-Infektion in AGMs reine Nebeneffekte der Infektion sind oder ob diese tatsächlich Auswirkungen auf die Pathogenese haben, wie z.B. ein Verhindern der Ablagerung von Immunkomplexen in lymphatischen Geweben. Gegenstand zukünftiger Studien sollte sein, die Korrelation dieser Phänomene mit den neu identifizierten Kandidaten zu untersuchen.

Summary

The natural hosts of simian immunodeficiency virus (SIV), which include African green monkeys (AGMs), do not develop AIDS despite high viral loads. SIVagm-infected AGMs lack antibodies against the viral Gag protein, which is immunodominant in pathogenic systems, such as rhesus macaques. They do, however, develop strong humoural responses to the viral Env protein. It has been hypothesised that this lack of Gag-specific antibodies contributes to the lack of disease in AGMs, as there is a lack of immune complex deposition in the lymph nodes of natural hosts, which is in contrast to heterologous host systems where the fine structure of the lymph nodes is destroyed. AGMs may therefore have evolved a tolerance to Gag to avoid these immunopathological events. Previous experiments to break the apparent tolerance to Gag were performed by immunising AGMs with SIVagm Gag protein or SIVagm *gag* DNA, both approaches unexpectedly leading to the induction of Gag-specific antibodies. Upon challenge, however, the titres of such antibodies dropped to undetectable levels, thereby apparently falsifying the hypothesis that AGMs have evolved a state of immunological "tolerance" to Gag. Instead, the results suggested an active suppression of the protein-specific response in AGMs. To bypass this phenomenon, it was initially intended to generate an adeno-associated virus vector expressing the gene for a recombinant anti-Gag antibody. Upon 'gene therapy' with this construct, anti-Gag antibodies produced artificially in muscle cells of AGMs would enter the circulatory system independent of the immune system.

However, evidence appeared that due to the trimeric form of the viral spike, the humoural response to Env is T-cell independent, whereas the secondary antibody response to Gag strongly depends on T-cell help. Regulatory mechanisms that suppress general T-helper cell activity would therefore have no effect on the induction of anti-Env antibodies in AGMs but would abrogate the anti-Gag response. It was therefore decided to obtain a systematic overview of immunoregulatory biomarker levels during the acute and chronic phase of infection to identify possible down-regulating mechanisms that result in the absence of anti-Gag antibodies in the natural hosts of SIV.

Fortunately, sequential samples taken before and during acute and chronic infection of both macaques and AGMs were available from a previous immunisation study. Luminex multiplex assays were performed to determine the levels of 17 different pro- and anti-inflammatory

markers in plasma. Distinct differences in the immune activation profiles of AGMs and rhesus macaques during the acute phase of SIV infection were identified. Rhesus macaques showed distinctly higher levels of immune activation upon SIV-infection than AGMs and there was a clear difference in the timing of peak cytokine elevations, with levels in the AGMs not only being lower, but also occurring later than in rhesus macaques. The different timing in elevations might be a critical important factor for the induction of 'tolerance' to SIV in AGMs. Real-time PCR assays were established for the detection of 34 different biomarker expression levels in the PBMCs of AGM and rhesus macaques, which now provide useful tools for characterising immune activation profiles at the mRNA level. To achieve this, it was necessary to sequence the mRNAs coding for the AGM biomarkers.

Polymorphisms in the gene coding for IRF-7, a molecule involved in Toll-like receptor 7 (TLR7) signalling have been hypothesised to be responsible for lower interferon (IFN) alpha responses (and hence reduced immune activation) in plasmacytoid dendritic cells (pDCs) of sooty mangabeys (SM), another natural host species for SIV. The AGM IRF-7 sequence was determined and compared with the SM, human and rhesus sequence. One polymorphism was found to be shared in AGMs and SMs, but different to humans and rhesus. In addition, it was shown that AGM pDCs, like those of SMs, have a reduced ability to secrete Type 1 IFN following stimulation with SIV compared to those of rhesus macaques.

Finally, a decrease in total IgG in AGMs and a trend towards an increase of total IgG in rhesus macaques over the course of SIV infection were demonstrated.

Ultimately, two strong candidates for the dampening of T helper cell activation and hence B-cell responses to T-cell dependent antigens (such as Gag) in AGMs have been identified: The PD1/PD-L1 induced anergy of T helper cells and the severely reduced capacity for innate memory B-cell activation by TLR7 activation, due to the diminished IFN alpha responses of AGM pDCs to SIV.

It is not yet known whether the phenomena of abrogated anti-Gag antibody production and reduced total IgG levels upon SIV-infection in AGMs are mere bystander effects of the infection or whether they indeed have implications for pathogenesis (such as avoiding the trapping of immune complexes in lymph nodes). Investigating these and their correlations with the candidates mentioned above should be investigated more fully in the future.

1 Introduction

1.1 The global AIDS epidemic and its emergence

The first cases of AIDS were reported in June and December 1981 [1, 2]. Two years later, the causative virus was first isolated in the laboratory of Luc Montagnier at the Pasteur Institute in Paris, France [3]. This discovery, awarded with the Nobel Prize in 2008 (To Luc Montagnier and Francoise Barré-Simoussi), was subsequently confirmed by other research groups around the globe [4, 5].

Since the discovery of HIV three decades ago, more than 60 million people have contracted the virus and more than 30 million have died of AIDS. Currently, approximately 34 million people are living with HIV [6]. Despite intensive efforts of education and prevention campaigns, there are still approximately 2.7 million new infections globally each year. The majority of HIV-infected people live in sub-Saharan Africa, where mainly young adults and children are affected. The spread of HIV in Eastern Europe is also alarming, with infection rates greatly exceeding the global average. Today it has become clear that HIV circulated in humans much earlier than the 80ies. For example, HIV has been detected in African plasma samples from 1959 [7] and phylogenetic analyses suggest infections as early as 1916 [8] (reviewed in [9]).

Various reasons for the sudden emergence of HIV-1 have been proposed. Major factors are considered to be the corporate, social and economic changes that took place in the first half of the 20th century [10]. The most common transmission routes for HIV are unprotected sexual contact and the sharing of needles between intravenous drug users.

1.2 Phylogeny of primate lentiviruses

The closest relatives to HIV are the simian immunodeficiency viruses (SIV), lentiviruses belonging to the family of retroviridae. One common feature of all retroviruses is the capacity for reverse transcription, i.e. the transcription of RNA into DNA. Human lentiviruses can be divided into two major groups, HIV-1 and HIV-2 that have a sequence identity of about 55% [11].

So far, SIV could be detected in more than 40 African primate species [12, 13]. These primates become infected with their species-specific viruses in the wild and are considered the natural hosts for SIV. Moreover, the infection has no pathogenic effect in these natural hosts, i.e. they do not develop AIDS. However, natural hosts for SIV carry a vast reservoir of lentiviruses that could potentially cause zoonoses in humans.

The following phylogenetic tree provides an overview of the major groups of primate lentiviruses (figure 1.2):

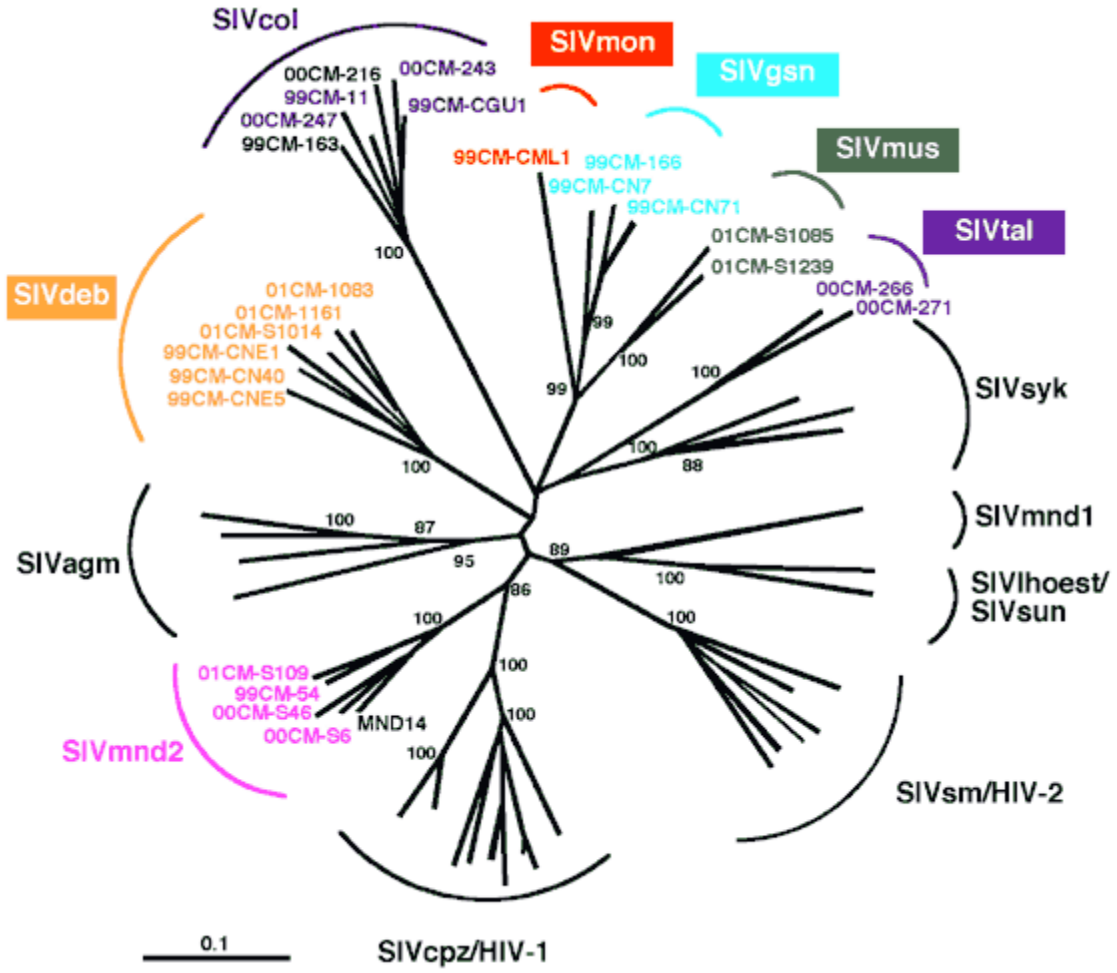

Figure 1.2: Subgroups of SIV: The most recently described groups are highlighted in colour. The phylogenetic tree is based on sequences of a 650bp fragment of the polymerase gene and their alignment is according to the neighbour joining method. The 0.1 bar represents a divergence of 10% (From: [12])

The different SIV sequences from each primate species cluster according to their respective host, i.e. the highest sequence identities exist between isolates within one species. This indicates a long phylogenetic co-evolution of SIV subspecies and their primate hosts.

If cross-species transmission of a primate lentivirus occurs, the virus can have a pathogenic effect in the new, heterologous host. If, on top of that, the virus is also transmittable to other individuals of the heterologous host species, an epidemic can occur. For example, inoculation of Asian rhesus and pig-tailed macaques with SIVsm (the virus naturally infecting African sooty mangabeys (SMs)) results in an infection that can be transmitted to other macaques and leads to the development of AIDS [14, 15].

The HIV-1 and HIV-2 epidemics both arose from cross-species transmission. The high sequence similarity indicates that SIVcpz from chimpanzees (Pan troglodytes troglodytes) is the ancestor of HIV-1. Therefore, the precursor of HIV-1 was probably transmitted from chimpanzees to humans, whereas SIVsm, showing the highest sequence similarity to HIV-2, was probably the precursor of the latter and transmitted to humans from SMs. Initial transmission of the viruses is most likely to have been a consequence of the trade in 'bush-meat', i.e the hunting and butchering of primates for their meat, which involves frequent contact with infected blood through open wounds or the mucosa [16]. This practice is common in many African countries and is therefore a plausible transmission route for HIV.

Sequence analysis of the env gene, coding for the viral envelope protein, is used as a basis for the systematic nomenclature of HIV-1 and HIV-2 isolates. For HIV-1, three groups (M, main; O, outlier; N, non-main/non-outlier) were defined. Isolates of group M are by far the most common in the global epidemic; they are further divided into subgroups A-K. Circulating recombinant forms comprising 'mixtures' of viruses from different subgroups also exist. Subtype B is the most prevalent In Europe and the USA whereas in Asia is subtype C and in Africa, all subtypes are prevalent. HIV-2 is mainly endemic in western Africa and is directly divided into subtypes A-E [17]. The existence of the groups M, N and O of HIV-1 suggests at least three independent cross-species transmission events and in the case of HIV-2, a minimum of four such events has been suggested [18].

1.3 Structure and morphology of HIV and SIV

Infectious particles of HIV and SIV possess the typical structure of retroviruses with a diameter of approximately 110nm (figure 1.3). The outer membrane originates from the cytoplasmic membrane of the previous host cell. The viral glycoproteins are associated with this membrane. The transmembrane glycoprotein gp41 is anchored in the membrane, whereas

gp120, the external glycoprotein, is non-covalently attached to gp41. These heterodimers form homotrimers on the membrane [19]. The so-called env-trimers facilitate the contact between virus and surface receptor on the host cell and are also responsible for the fusion of virus and cell membrane. The inner surface of the viral membrane is coated with matrix proteins (p17) that are attached to the membrane via myristic acid residues. In lentiviruses, the latter form trimers that attach to each other forming an icosahedric structure. Inside the matrix resides the conical virus capsid or core, consisting of capsid proteins (p24 in HIV-1, p27 in SIV) and surrounds the viral RNA genome, which in turn builds complexes with nucleocapsid proteins. Additionally, the viral enzymes reverse transcriptase (RT), integrase and protease are located within the core [20].

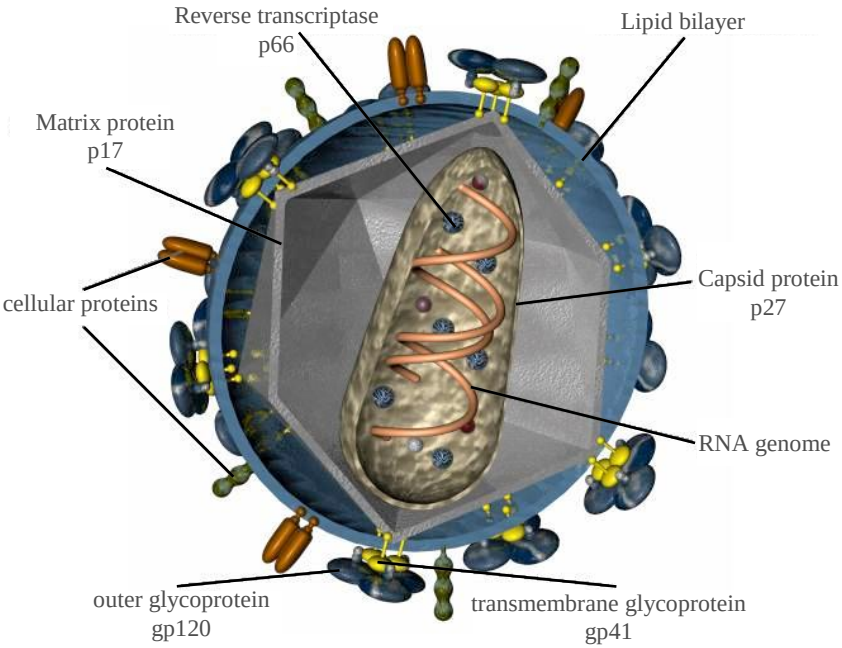

Figure 1.3: Schematic illustration of an SIV particle. Courtesy of Dr. S. Norley.

The lentiviral genome consists of two identical RNA strands with positive polarity that are both approximately 9.7kb long. There is no association through covalent bonds or base-pairing between the two. Both RNA molecules possess the typical features of eukaryotic mRNA, i.e. a 7-methyl guanine residue on the 5' end and a polyadenylated 3' end.

The terminal regions of the viral RNA contain regulatory elements that are responsible for the integration of the viral genes into the host cell genome and for regulation of transcription after integration has taken place. A tRNA that originates from the previous host cell is attached via

pairing of complementary bases to each viral mRNA. This tRNA serves as primer molecule for the initiation of reverse transcription.

The genomes of HIV and SIV contain three genes: gag (group specific antigen), pol (polymerase) and env (envelope). Gag codes for a precursor protein that is further spliced into the nucleocapsid proteins, the capsid protein p24 (or p27, respectively) and the matrix protein p17 by the viral protease. Pol codes for another precursor protein that is further spliced into the three enzymes RT, integrase and protease. Env codes for the precursor (gp160) of the glycoproteins gp120 and gp41. The proteolytic cleavage of gp160 occurs during the budding of new virus from its host cell.

Lentiviruses exhibit the two regulatory genes tat and rev. These regulate the transcription of viral RNA, alternative splicing of the viral genome and the export of viral mRNA from the nucleus. Primate lentiviruses possess four additional accessory genes: nef, vif, vpr and vpu (HIV-1) or vpx (HIV-2 and SIV), respectively. The proteins these accessory genes code for are not essential for viral replication in vitro, but have an impact on replication competence and pathogenicity [21, 22]

1.4 The replication cycle of HIV and SIV

HIV is naturally transmitted during unprotected sexual intercourse (anal or vaginal) or between a mother and her child during pregnancy, childbirth and breastfeeding. Infection can also occur as a result of sharing contaminated needles (usually by intravenous drug users) or by transfusion of contaminated blood or blood products (nowadays virtually eliminated by screening of blood donations). SIVs are thought to spread between primates in the wild by sex, through blood-to-blood contact during fights and, in the case of cross-species transmission, as a result of predation.

Once the virus has entered the body, adsorption of the virus to the target cell is facilitated by the external viral glycoprotein gp120. In addition to CD4, chemokine receptors of the CC- or CXC-type are used as coreceptors for viral entry [23, 24]. The natural ligands for CCR5, the main coreceptor, are RANTES, MIP-1alpha and MIP-1beta, members of the beta-chemokine family. Beta-chemokines are secreted by $CD8^+$ T-lymphocytes and have been demonstrated to inhibit HIV replication in vitro [25]. In the early phase of infection, viruses that use CCR5

(R5-viruses) as coreceptor dominate the population. Later in the symptomatic stage of infection, virus tropism can switch from CCR5 to CXCR4 (fusin) to give 'X4-viruses' although this is dependent on the viral subtype and is not always observed. Other chemokine receptors of the CCR and CXCR family have also been identified as coreceptors for HIV and SIV [26, 27]. Binding of gp120 to the cellular surface receptors CD4 and CCR5 induces a conformational change that leads to the fusion of virus and cell membrane, thereby allowing the entry of the RNA-containing cores into the cytosol.

After the release of the viral capsid into the cytosol, it is unpacked and the viral RNA is released together with the enzymes responsible for reverse transcription and integration. Using the tRNA from the previous host cell as primer, the RNA is reverse transcribed into DNA by RT, yielding a DNA/RNA hybrid [28]. Because RT has no 3'-5' exonuclease activity (proof-reading), the mutation rate is relatively high, causing high variability within the genome [29]. Following reverse transcription, the DNA/RNA hybrid is hydrolysed by the RNase H activity of RT and subsequently converted to double-stranded DNA that is transported into the nucleus to be randomly integrated into the host cell genome by the action of the viral integrase. The integrated viral genome (provirus) can now be passed on to all daughter cells by mitosis.

The provirus is flanked by long terminal repeats (LTRs) consisting of the regions U3, R and U5. These are arranged in the same orientation at both ends (figure 1.4).

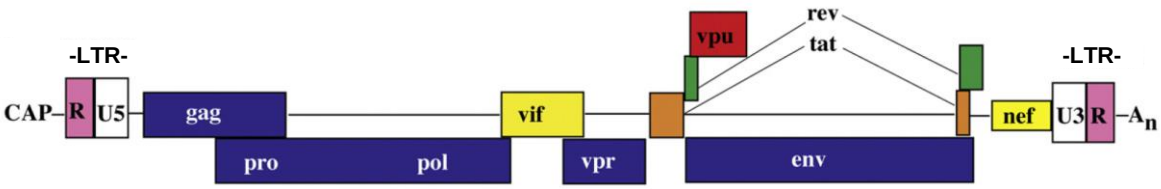

Figure 1.4: The proviral genome of HIV-1. R: redundant region. U3 and U5: unique regions at the 3'- and 5'- end, respectively. LTR: long terminal repeats. CAP: 7-methyl guanine residue on the 5' end. A_n: polyadenylated 3' end. Purple: regions coding for structural proteins and enzymes. Orange and green: regions coding for regulatory genes tat and rev, respectively. Yellow: regions coding for virion infectivity factor (vif) and negative regulatory factor (nef). Adapted from: [30]

All genes of the proviral genome are under the control of the promotor localised in the 5' LTR. In addition, various cellular receptors, facilitating transcription of provirus by the cellular RNA polymerase II, can bind to the U3-region. The most important of these are SP1

and NFkB, the latter being activated by T-lymphocytes upon infection. The activation stage of the host cell therefore determines whether virus will be actively transcribed or become "dormant" in a latent state [31]. The initially transcribed RNA is approximately 9kb and can be alternatively spliced into more than 30 different mRNAs [32]. Amongst others, this results in the generation of the regulatory proteins Tat, Rev and Nef that are factors for *in vivo* replication. Tat and Rev are responsible for regulating the generation of full-length and single-spliced RNA, respectively. Full-length RNA, transcribed from the integrated provirus by cellular RNA polymerase II, is either used as genomic RNA for the production of new virus particles or for the synthesis of Gag and Pol precursor proteins. Like the viral RT, RNA polymerase II lacks proof-reading activity and is therefore contributes to the high variability of HIV and SIV. Single-spliced viral RNA provides the basis for the translation of vif, vpr, vpu/vpx and env. The viral protease cleaves Gag and Pol precursor proteins into functional units and packaging and self-aggregation of the structural proteins occurs at the cellular membrane. Newly formed virus capsids exit the cells via exocytosis (so-called "budding" of the virus), by which they acquire from the host cell a lipid membrane with integrated viral glycoproteins.

1.5 Pathogenesis of HIV/SIV infection in the heterologous host

HIV/SIV infection of hetrologous hosts can be divided into three stages. The first is the acute infection phase lasting between two and six weeks during which about 50% of all infected individuals experience flu-like symptoms [33]. Spread of the virus from the site of entry to lymphatic tissues can occur within a few days [34], allowing a correspondingly rapid detection in the lymph nodes. The main targets for viral replication are the activated CD4+ T-cells in the gut-associated lymphoid tissue (GALT) [35]. However, during the course of infection, other T-cells, microglia cells and macrophages also become infected [36]. Plasma virus loads (pVL) peak at 10^4-10^8 virus particles per millilitre approximately two weeks after infection and the cell-associated virus load (integrated provirus) peaks at 10^2-10^4 infected cells per million peripheral blood mononuclear cells (PBMCs). At this time, a dramatic decrease in $CD4^+$ T-cells occurs and although there is a slight rebound after the acute phase, counts never again reach the pre-infection levels [37-39].

The transition to the asymptomatic phase begins with the emergence of antiviral immune responses, particularly virus-specific CTLs. Plasma virus loads accordingly decrease but are

eventually maintained at about 10^3-10^5 particles per millilitre [40]. This so-called set-point virus load is one quantitative indicator for disease progression [40-42].

After an average of 8-10 years, the infection progressed to the third stage, the symptomatic phase. This is characterised by a renewed decrease in circulating CD4+ T-cells 200 per microliter blood or less. The morphologic tissue structure in the lymph nodes disintegrates and levels of virus-specific antibodies and CTLs decrease.

The progressive weakening of the immune system leads to infections with opportunistic pathogens that are normally harmless in immunocompetent individuals. In immunosuppressed AIDS patients, however, they can cause severe infections that are extremely difficult to treat. The most common illnesses include pneumonia caused by *Pneumocystis carnii (PCP)*, mucosal candidiases, tuberculosis and severe virus infections, usually cytomegalovirus and herpes zoster [43]. Furthermore, aggressive forms of Karposi's sarcoma or B-cell lymphoma are common and infections of microglia cells can cause neurodegenerative disease. Lacking an effective immune system, the patitent eventually succumbs to infection and dies [44-47].

1.6 Immune responses in the heterologous host

1.6.1 Cellular immune reactions against HIV/SIV

Cytotoxic T-lymphocytes (CTLs) recognise and kill virus-infected cells and make a major contributing to clearing infections [48]. The cells recognise peptides of 8-12 amino acids expressed on the surface of infected cells by MHC class I molecules. The T-cell receptor binds to the complex of MHC-I molecule and cognate peptide [49] and initiates the formation of an immunological synapse [50]. A number of proteins, perforin, granzyme and granulysin are then released across the synapse to enter and induce apoptosis of the infected target cell while sparing adjacent cells.

In HIV-infection, virus-specific CTLs can be detected as early as 1 week p.i. [39, 51] and the increase of CTL responses during the acute infection phase inversely correlates with pVL [52-54]. In heterologous host systems such as humans and rhesus macaques, virus specific CTL responses are remarkably strong [55, 56]. However, as is often the case for epitopes

recognised by antibodies, CTL epitopes can also undergo escape mutation [57] with the mutated epitopes no longer able to bind to the MHC complex, or not recognised by the CTL.

Despite some CTL epitopes being located within highly conserved regions of HIV proteins and therefore unable to 'escape' [58], the responses are still unable to completely eliminate all HIV-infected cells. There are a number of possible reasons for this. First, downregulation of MHC expression by the viral Nef protein in infected cells will render the cell 'invisible' to CTLs. Second, the virus may replicate at sites in the body to which CTLs have no access. Third, the CTL response may be too slow to prevent the release of at least some virus particles before killing occurs. Finally, there are indications that limitations in the development and activity of HIV-specific CTLs compared to CTLs specific for other viruses play a role [59].

1.6.2 Humoural immune response to HIV/SIV

Seroconversion, i.e. the development of specific antibodies against HIV/SIV takes place within the first three months post infection (p.i.). Very often, antigen-specific antibodies can be detected as early as two weeks p.i. During this acute phase of infection, most antibodies that develop will be directed against epitopes of the structural proteins Gag and Env. Some of the antibodies generated may have also neutralising activity against HIV/SIV [60-62]. Antibodies specific for the HIV-1 envelope glycoprotein appear at approximately three weeks p.i. and those that neutralise are directed against three main regions of Env (reviewed in [63]).

The first described broadly reactive neutralising antibody (BrNAb), b12, was isolated from a clade B-infected patient in 1992. This binds to the CD4 binding site of gp120 and is able to neutralise approximately 50% of clade B viruses and 30% of non-clade B viruses [64]. The monoclonal antibody 17b binds to the coreceptor binding region (CD4-induced region), an epitope highly conserved across all HIV-1 clades. Unfortunately, for two main reasons this antibody shows only weak neutralisation activity against most primary isolates of HIV-1 [65]. First, following viral attachment the epitope is only briefly exposed, giving the antibody a very short time to act. Second, because the cleft 17b has to fit into is deep and very narrow, steric hindrance probably limits its access to the epitope. 2F5, 4E10 and Z13 are all BrNAbs binding to the membrane proximal external region (MPER) of gp41. Their binding is thought to interfere with viral fusion, but the emergence of such antibodies is relatively rare during

HIV infection. It has been suggested that mechanisms of self-tolerance select against MPER-reactive B-cell clones, because MPER epitopes appear to share homologous regions with self-proteins like cardiolipin [66]. 2G12 is a BrNAb that binds to a complex set of glycans on the gp120 surface and is broadly reactive against many clade B viruses, but the breadth of reactivity decreases against non-clade B viruses [67]. PG9 and PG16 are closely related somatic mutants of a BrNab that was isolated from an HIV-infected African donor. These bind to novel epitopes at the apex of gp120 (V2 and V3 loop) and potently neutralise about 75% of known HIV-1 strains. Indeed, they are the most broadly reactive NAbs isolated to date [68].

All neutralising antibodies so far isolated and studied have been derived from clade B HIV-infected individuals and they are usually only generated after several months of infection, resulting nevertheless in immune pressure on the virus. This is complicated by successive waves of viral escape from the autologous virus, driven by the generally high mutation rate [69, 70] and particularly the hypervariability of the HIV V3 loop [71-73]. Such escape mutants [74-77] develop rapidly enough to ensure continued high-level viral replication and therefore too quickly for the antibodies to have a real impact on virus load [78]. However, 'escaped' viruses generally remain sensitive to BrNAbs such as b12 and 2G12 or MPER-directed BrNAbs, showing that the initial antibody response is not directed to these more conserved epitopes. One of the reasons for this is the heavy glycosilation of Env proteins that facilitate the virus to “shield” possible epitopes. Another problem is the readily dissociation of gp120 from gp41, the so-called "shedding" of the virus. It leads to the exposure of regions of gp41 that are not exposed on mature functional spike, which are nevertheless highly immunogenic. Thus, most HIV-infected patients show high titers of antibodies against gp41 that are unable to bind to the native virus spike [79-81].

Functional antiviral effects mediated by antibodies are not limited to direct neutralisation of the virus particle. It has, for example, long been known for other infections that activation of complement by antibody bound to the virus or virus infected cell can result in virolysis or complement-mediated lysis, respectively [82]. However, these functions appear to play only a minor role for HIV [83-85], probably due to the fact the viral membrane (and areas of the infected cell express the viral Env as a prelude to budding) preferentially incorporates proteins capable of blocking the complement cascade, such as CD46, CD55 and CD59 (Marschang [86-88].

A third form of antibody-mediatd immune response is antibody dependent cellular cytotoxicity (ADCC; reviewed in [89]). This relies on the specific binding of antibodies to viral proteins presented on the surface of infected cells, which initiates the killing of the "marked" cells by killer cells, such as NK cells, monocytes or neutrophils, recognising the Fc-region of the attached antibodies [90, 91]. This has also been demonstrated for HIV in vitro [[92], [93], [94]]. Antibodies facilitating ADCC are mainly reactive against Env, but recent studies have also suggested a role for antibodies against Nef, Vpu and Pol (Yamada [95, 96]. There is strong evidence for an inverse correlation between ADCC activity and clinical disease in HIV-infected individuals and SIV-infected rhesus macaques [97, 98].

In addition to their possibly beneficial roles in neutralisation, virolysis and ADCC, Env-specific antibodies can, under certain conditions, enhance rather than inhibit virus infectivity [99], a phenomenon also shown for retroviral infections of horses, cats and monkeys [100-102].

1.6.3 Immune activation

Probably the most detrimental characteristic feature of pathogenic HIV/SIV infection is the establishment of a state of chronic and generalised immune activation (reviewed in [103]). The latter is characterised by high serum levels of proinflammatory chemokines and cytokines [104-106], high levels of activated and proliferating T-cells [107-109] and an increased susceptibility of uninfected T-cells to undergo activation-induced apoptosis [110-113]. The level of chronic immune activation appears to be the strongest correlate of disease progression in pathogenic HIV/SIV-infection of heterologous hosts. In particular, it is becoming increasingly clear that the events during the early acute phase of pathogenic HIV/SIV infection play an important role in "fuelling" immune activation and viral spread. During acute HIV infection a dramatic cytokine "storm" occurs, a phenomenon not seen in acute hepatitis B (HBV) or C (HCV) infection, for example [114]. Some cytokines are upregulated as pVL increases while others peak as early as seven days after infection (Note: the functions of all cytokines and chemokines mentioned in this thesis are listed in the appendix). IFN-alpha levels rapidly increase to their highest levels within the first day, associated with an early increase of TNF-alpha. Other cytokines and chemokines that are upregulated within the first wave include IL-15, IP-10 and MCP-1. A second group that includes IL-6, IL-8, IL-10

and IFN-gamma become elevated during the second wave at approximately 7 days p.i. The third wave occurs 9-12 days p.i., when IL-4, IL-5, IL-12 and IL-22 become elevated. This cytokine "storm" occurs during acute HIV infection, but does not appear to occur during HBV and HCV infection. Since HBV and HCV only persist in about 10% and 60-80% of infected individuals, respectively [115], it has been suggested that a rapid induction of a robust cytokine response is not necessarily a requirement for the prevention of virus persistence [114]. In fact, high levels of proinflammatory cytokines and chemokines during acute influenza A (H5N1) and severe acute respiratory syndrome (SARS) infections are associated with an enhanced pathogenesis [116, 117]. Likewise, the strong cytokine response during acute HIV infection could contribute to the early immunopathology of infection as well as to its long-term consequences.

1.7 Long-term non-progressors, highly exposed persistent seronegatives and elite controllers

Cytotoxic T-cells produce MIP-1alpha, MIP-1beta and RANTES, the secretion of which is not restricted to the recognition of MHC-complexes. All of these chemokines are able to dampen HIV replication in vitro by competing for the binding of CCR5. IL-16, a chemoattractant cytokine, is also able to restrict HIV-replication, most likely by restricting HIV transcription [118-120]. Various studies have demonstrated high levels of these factors in individuals who have survived a long time without treatment, so-called long-term non-progressors (LTNPs) [121], who account for 2-15% of HIV-infected individuals, depending on the study population [38, 122-124]. Other factors for the restriction of *in vivo* replication have also been suggested [125, 126]. Another group of patients that are apparently able to avoid disease progression are the elite controllers (reviewed in [127-129]), though the definitions for elite controllers and LTNPs often overlap. The latter usually present low to moderate levels of viremia [42, 123, 130-132], whereas in elite controllers, the plasma virus load can be hardly detectable. The prevalence of elite controllers is below 1% [124, 133-135] and the majority of them possess very strong $CD8^+$ T-cell responses against HIV-infected cells, which are lysed as a result of perforine secretion. This demonstrates that $CD8^+$ T-cells play indeed a very important role in the suppression of virus replication in the pathogenic system. Some MHC-types, such as HLA B57 and B27 are highly represented amongst elite controllers and they have been shown to present epitopes that are highly conserved within the viral genome [134, 136, 137]. A very small group of individuals has even been shown to

apparently resist HIV infection, despite having been exposed to the virus. These highly exposed persistent seronegatives (HEPS) feature a lack of antibodies against HIV and very often no detectable viraemia [138-140]. In some HEPS, mucosal antibodies and CTLs against HIV could be demonstrated [141-143]. This is particularly interesting, since the emergence of CTLs cannot have happened without prior infection, thus, it could be that CTL responses had been sufficient to clear the infection in these patients. However, as testing methods have been increasing in sensitivity, small amounts of HIV could be detected in some HEPS [144].

1.8 Antiretroviral therapy

The first success in the treatment of AIDS was achieved in 1987 when Azidothimidine (AZT), a nucleoside analogue inhibiting the function of RT, was licensed. Unfortunately, not only was use of the drug accompanied by severe side effects but it also became quickly clear that the success of such monotherapy was very short-lived due to the virtually inevitable development of resistant mutants [145-147]. Modern treatments usually involve a combination of different inhibitors typically directed against both reverse transcriptase and the viral protease, a strategy termed 'highly active antiretroviral therapy' (HAART). The probability of a mutant occurring that is simultaneously resistant to three different drugs is extremely low and in most cases, treatment with HAART results in prolonged suppression of pVL below the detection limit although. However presumably due to the reservoir of silent proviruses in the body, such treatment can never clear the infection and discontinuation usually results in a rapid rebound in pVL. Individuals with HIV/AIDS therefore need to take HAART continuously for their entire lives and it is still too early to be sure that HAART can fully prevent progression to disease rather than just delay it. A major problem with a treatment regime comprising of multiple drugs taken daily for many years are the costs. In 2002, this was estimated to be €24,000 per year [148], which although sustainable for developed countries with a modern health care infrastructure and low numbers of patients, is clearly out of reach for the majority of developing countries afflicted most severely with the disease. Since then, the mean prices of first-line antiretroviral medicines have been reduced and low-income countries can now provide a year of antiretroviral therapy at a median cost of US$ 137 per person [149]. However, the majority of HIV-infected people in the world have no or only limited access to therapy. In 2009, only about 5.2 million people were being treated worldwide, which was estimated to be 35% of those in need [149].

1.9 Non-pathogenic SIV infection of natural hosts

African non-human primates (NHP), such as sooty mangabeys or African green monkeys (AGMs) are natural hosts for SIV, i.e. a large proportion of animals in the wild are infected with the virus. So far, SIV infections in more than 40 distinct African NHP have been reported [12, 13]. The major distinguishing characteristic of such infections of natural hosts compared to HIV infection of humans is the lack of disease development. This is a feature of the host, not the virus, as isolates from natural hosts can induce AIDS in a heterologous primate species. For example, infection of Asian pig-tailed macaques (Macaca nemestrina) with SIVagm from AGMs will result in the development of AIDS and infection of rhesus macaques (Macaca mulatta) with SIVsm from sooty mangabeys will do the same. Indeed, it was the accidental infection in a primate centre of Asian macaques with SIVsm that yielded SIVmac, the virus most commonly used in macaques for AIDS vaccine development [14, 150-153]. Finally, SIVsm is also known to be the origin of HIV-2 (reviewed in [154]). Elucidating the underlying biological reasons for the natural host's resistance to disease despite long-term SIV infection would not only help us understand the mechanisms of pathogenesis in HIV-infected humans but might also provide new avenues for therapy. A great deal of research has been carried out in this area, the results of which will be summarised below.

1.9.1 Levels of virus replication

The fact that natural hosts for SIV do not develop AIDS (but heterologous hosts infected with the same virus do) initially suggested that the immune system plays an important role in the outcome. Since natural hosts are able to maintain CD4+ T-cell counts that are similar or only slightly lower than the levels found in their uninfected counterparts [155-161], their preservation of immune competence is not surprising. However, a number of studies demonstrated that this phenomenon is in fact not due to reduced levels of viral replication [161-164]. In fact, the levels and kinetics of virus replication in natural hosts are remarkably similar to those observed in pathogenic HIV/SIV infection (figure 1.9.1 below, [165-167]. Although it has been widely assumed that AIDS is a direct result of prolonged, high-level virus replication, the high viremia observed in the natural hosts for SIV with no resulting pathogenicity indicates that other factors are important.

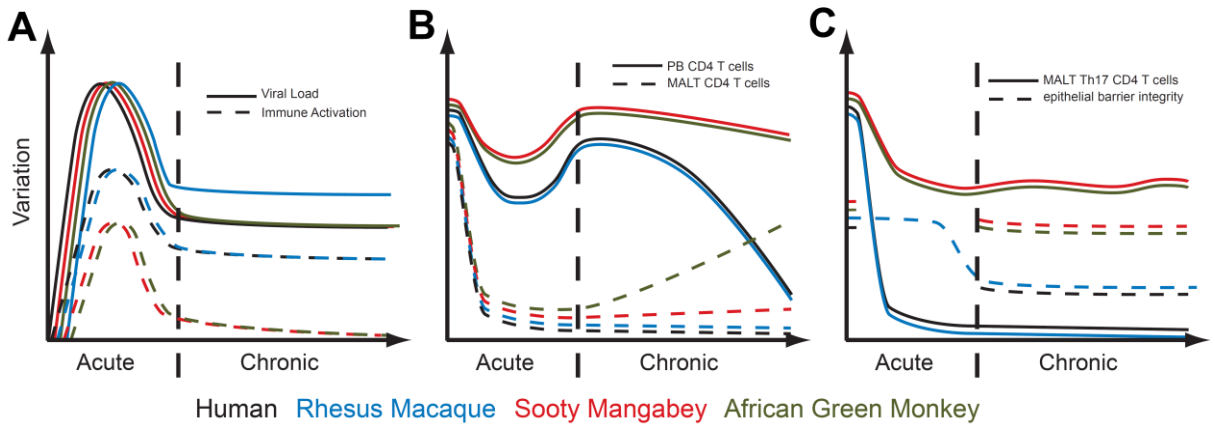

Figure 1.9.1: Virologic and immunologic markers of HIV/SIV infection during the course of pathogenic HIV/SIV infection in humans (black lines), rhesus macaques (blue lines) and nonprogressive SIV infection in sooty mangabeys (red lines) and African Green monkeys (green lines).

(A) Levels of viral replication (solid lines) and immune activation (dotted lines), defined as levels of T- and B-cell activation/proliferation, Interferon stimulated gene response, type I interferon production.
(B) Levels of $CD4^+$ T-cells in peripheral blood (solid lines) and gastrointestinal tracts (mucosa-associated lymphoid tissue [MALT], dotted lines).
(C) Levels of mucosal Th17 cells (solid lines, defined as IL-17–producing CD4 T cells) and integrity of the epithelial barrier at the mucosal level (dotted lines). Data on epithelial barrier integrity during the acute phase of infection are available only for RM.
Adapted from: [168]

In contrast to the situation in pathogenic systems, there is no correlation between $CD4^+$ T-cell counts and pVL in natural hosts for SIV [156, 158]. This could indicate that (all other things being equal) the primate immune system might maintain its normal function and counteract the persistent depletion of $CD4^+$ T-cells.

1.9.2 Levels of mucosal CD4+ T-cells

Since natural hosts for SIV do in fact exhibit a rapid and severe depletion of mucosal CD4+ T-cells upon infection (figure 1.9.1 above, [169]), it is clear that the infection is not without impact. In fact, similar to the situation with plasma virus load, the magnitude and kinetics of acute mucosal CD4+ T-cell depletion in natural hosts for SIV are strikingly similar to that seen in SIV-infected macaques [170-172], although in the latter depletion is associated with systemic immune activation and increases in severity as the animal progresses to AIDS. In both SMs and AGMs, a correlation between mucosal CD4+ cell depletion and systemic immune activation could not be demonstrated [165, 169]. This preservation of mucosal immunity in natural hosts for SIV is consistent with another observation that distinguishes pathogenic from non-pathogenic infection: In pathogenic HIV/SIV infection, high levels of

microbial translocation from the intestinal lumen to the systemic circulation can be observed [173], a phenomenon not detectable in SIV-infected natural hosts [169]. This suggests that, despite significant depletion of mucosal CD4+ T-cells, natural hosts for SIV are able to maintain an intact mucosal barrier. The idea that CD4+ T-cell depletion alone determines progression to AIDS is therefore too simplistic to explain the disease in humans.

1.9.3 Target cells for SIV replication and cytopathicity

Even though SIV infections of natural hosts are associated with high levels of viraemia, this could still be associated with normal CD4+ T-cell counts if the viruses were targeting cell types different than those in heterologous hosts or was less cytopathic in these animals. However, the majority of infected cells in SMs and AGMs are indeed CD4+ lymphocytes (reviewed in [174]) and experimental *in vivo* depletion of CD4+ T-cells leads to a marked decrease in pVL in SMs [175]. This rebounds with the increase of activated CD4+ T-cells, suggesting that the major determinant of set-point viraemia in SIV-infected SMs is indeed the availability of target cells. In addition, it has been shown that in both SMs and AGMs, 92%-99% of viral replication is sustained by short-lived cells with an average lifespan of 1.1 days and that these cells die as a result of direct cytopathic effects of the virus [176]. Taken together, these results show that natural SIV infections are remarkably similar to those of heterologous hosts in terms of target cell type, depletion of CD4+ T-cells and direct virus cytopathicity. This in turn suggests that additional cofactors are critical in the pathogenesis of heterologous HIV/SIV infection.

1.10 Immune responses in natural hosts of SIV

1.10.1 Cellular responses

The observation that the level of set-point viraemia in the natural hosts for SIV is comparable to that observed in pathogenic HIV/SIV infection strongly suggests that the resistance to AIDS is unlikely to be the result of a stronger or more effective immune response to the virus. Correspondingly, even though low levels of virus-specific CTL responses have been demostrated in AGMs ([177, 178] and Siegismund et al., submitted), many studies support the

hypothesis that SIV-specific CTL responses are not a major determinant of set-point viraemia in natural SIV infection [179].

1.10.2 Humoural responses

Earlier studies in our group demonstrated that even though infected AGMs develop very high titres of antibodies against whole SIVagm lysate (consisting mainly of Gag and Env proteins), no antibodies against the purified viral Gag protein could be detected [180]. This was somewhat surprising, since in the heterologous host systems, very high titres of such antibodies are induced. In contrast, pig-tailed macaques infected SIVagm do develop strong anti-Gag responses, suggesting that their apparent lack in AGMs is truly a feature of the host and not of the virus. Furthermore, a review of the literature revealed that in many other natural host systems the antibody response to Gag was also weak or absent (Table 1.10.2). It was therefore proposed that the natural hosts for SIV have developed over millennia of co-evolution a form of selective tolerance to the viral Gag protein. The advantage of this selective lack of antibodies was hypothesised to be the avoidance of the formation of Gag-antibody immune complexes that would become "trapped" in the lymph nodes during chronic infection, leading to complement activation and therefore to the destruction of lymphoid tissues. Indeed, during 'pathogenic' infections, trapped virus can be detected in the lymphoid tissue and there is a gradual degradation of lymph nodes architecture [181].

Table 1.10.2: Comparison of humoural responses to Gag protein in different immunodeficiency virus/host systems. Adapted from: [180]

Virus	Species	Strong anti Gag response	Trapping of Virus in lymph nodes	Pathogenic
SIVagm	African green monkeys	no	no	no
SIVsm	Sooty mangabeys	no	no	no
SIVmnd	Mandrills	no	-	no
SIVsyk	Sykes monkey	no	-	no
SIVmac	Rhesus macaques	yes	yes	yes
HIV-1	Humans	yes	yes	yes

As the neutralising antibody response to HIV in infected humans is relatively poor, it seemed possible that the natural hosts had the capacity to mount a stronger response able to maintain control of the virus. However, only minimal neutralisation against both concurrent and previous virus isolates of SIV has been detected in both SMs and AGMs (reviewed in [180, 182]).

1.10.3 Efforts to break the apparent tolerance to Gag

If the lack of Gag-specific antibodies is indeed the reason for the lack of disease in the natural hosts, the obvious experiment to perform would be to induce such antibodies prior to infection. Such experiments had indeed been previously carried out by our group. In the first study, AGMs were immunised purified SIVagm Gag protein in the presence of a strong adjuvant in an attempt to break the apparent tolerance before infection [183]. Surprisingly, Gag-specific antibodies could be readily induced at high titres, but rather than being boosted anamnestically as expected, antibody levels dropped steadily upon infection with SIVagm but could be again boosted by a further immunisation with purified Gag protein. In contrast to that, all AGMs developed high titres of antibodies to whole virus lysate containing Env upon infection.

It was hypothesised that the exogenously administered protein was not being presented to or recognised by the AGMs' immune system in the same manner as endogenously produced protein during infection, which would explain the failure of the immunisations to prime the response to infection. To address this, a second study was performed (Siegismund et al., submitted) in which AGMs and rhesus macaques were bioballistically immunised with codon-optimised SIVagm or SIVmac Gag DNA in the presence of species-specific GM-CSF DNA as adjuvant. In contrast to the previous study, the immune response would therefore be primed by endogenously synthesised Gag, produced and presented in the same manner as during infection. However, whereas all rhesus macaques showed a vigorous anamnestic response upon SIVmac infection, once again no such response in AGMs occurred, despite the induction of Gag-specific antibodies (albeit at low titres and transiently) by DNA-immunisation. As observed previously, all infected animals developed high titres of antibodies to the whole virus lysate containing Env.

In summary, both studies appeared to falsify the initial hypothesis that AGMs have evolved a state of immunological "tolerance" to Gag and the second study falsified the hypothesis that differences in the route of presentation was responsible for the lack of anamnestic response upon infection. Instead, the results suggested an active suppression of the protein-specific response during infection of the natural host, a phenomenon not observed in the heterologous macaque system.

1.10.4 Immune activation in the natural hosts

As described earlier, one of the most dramatic features of HIV infection of humans (and SIVmac infection of macaques) is the profound and prolonged state of hyperimmune activation (reviewed in [103]). In sharp contrast, a low level of immune activation is a key feature of natural SIV infection, despite high levels of virus replication [156, 158, 159, 162, 166, 184]. Several studies have demonstrated dramatically lower levels of cells expressing markers for activation and proliferation in SIV-infected natural hosts compared to SIV-infected macaques [160, 162, 165, 166, 174, 184-186]. Consistent with this, levels of bystander apoptosis are lower than in the pathogenic systems [187] and T- and B-cell turnover rates are not elevated in SIV-infected natural hosts compared with uninfected animals [188]. The cellular and molecular mechanisms responsible for avoiding chronic immune activation in natural hosts for SIV are still poorly understood. Several mechanisms have been proposed, including a lack of microbial translocation from the intestinal lumen to the blood circulation, a phenomenon that might abrogate Toll-like receptor (TLR) mediated activation of macrophages and dendritic cells [173, 189]. It has also been proposed that the Nef protein of the viruses in the HIV-1 lineage has lost its ability (evolved in 'natural' SIV) to downregulate CD3-TCR complexes on the surface of infected cells, a function that would normally reduce the level of T-cell immune activation [190]. In AGMs, an increase in regulatory T-cells (Tregs) has been documented within the first 24 hours of infection and during the subsequent acute phase, suggesting that Tregs play a role in inducing an anti-inflammatory milieu (increased plasma levels of TGF-beta and IL-10) that could contribute to the attenuated immune phenotype in non-pathogenic infection [184]. However, similar increases and temporal changes in markers for Tregs were not consistently associated in sooty mangabeys with resolution of immune activation during acute SIVsm infection [191]. Instead, a rapid increase in the cell surface marker programmed death 1 (PD-1) in the lymph nodes of acutely infected SMs was observed that correlated closely with immune downregulation. PD-1 is expressed on CD4+ and CD8+ T-cells, NK-cells, B-cells and activated monocytes and transduces an inhibitory signal when engaged simultaneously with TCR-signalling. Therefore, the level of PD-1 expression and the extent of ligand engagement regulate the threshold for T-cell activation. An increase in PD-1 was also detectable in SIVmac-infected macaques, but only at two weeks post infection, when levels in infected sooty mangabeys had already dropped back to baseline. This raises the possibility that AGMs and SMs have evolved different strategies (Tregs versus PD-1) to avoid hyperimmune activation, although it must still be ruled out that the apparent discrepancy in terms of Treg upregulation in the two studies

was simply due to differences in methodology. Nevertheless, it is becoming increasingly clear that an essential factor in the immune activation or downregulation in pathogenic and natural host systems is not just the type of response but also the timing.

1.13 Aim of the thesis

In the previous studies involving immunisation of AGMs with SIVagm Gag or DNA coding for Gag protein, it was not possible to determine whether the down-regulation of specific antibodies upon infection was protein-specific, virus-specific or systemic. The fact that infected AGMs developed high-titre antibodies to the Env glycoprotein despite losing the responses to Gag suggested that the lack of Gag-response is protein-specific. Whatever the mechanism, it was not possible to induce by immunisation the prolonged, high levels of Gag-specific antibodies concurrently with infection needed to test the hypothesis that antibody/Gag complexes contribute to disease in heterologous hosts. To overcome this problem, it was initially intended to “force” AGMs to produce anti-Gag antibodies throughout the entire course of SIVagm infection by generating an adeno-associated virus (AAV) vector expressing the gene for a recombinant anti-Gag antibody. Upon 'gene therapy' with this AAV construct, anti-Gag antibodies produced artificially in muscle cells would enter the circulatory system independent of the immune system.

However, evidence appeared that due to the trimeric form of the viral spike, the humoural response to Env is T-cell independent [192], whereas the secondary antibody response to Gag strongly depends on T-cell help. Regulatory mechanisms that suppress T-helper cell activity would therefore have no effect on the induction of anti-Env antibodies in AGMs. This raised the possibility that the lack of anti-Gag antibodies in AGMs is just one aspect of a generalised dampening of the T-cell response during early infection, a phenomenon that would not influence the T-dependent response to Env. Such an early (and sustained) suppression of T-cell activation could therefore be the critical event preventing the establishment of chronic hyperimmune activation. It was therefore decided to take a step back and perform a systematic overview of immunoregulatory biomarker levels during the acute and chronic phase of infection. This would involve the development and use of assays to measure and compare the levels of different pro- and anti-inflammatory markers in pathogenic (macaque) and natural (AGM) hosts systems. Such detailed knowledge of the early-phase and chronic immunological responses in pathogenic and natural host systems should provide insight into the biological mechanisms that result in the absence of anti-Gag antibodies and allow the

natural hosts of SIV to avoid the states of profound hyperimmune activation and hence the progression to disease.

2 Materials and Methods

2.1 Sequencing of the AG3.0 heavy and light chain variable regions

RNA was isolated from 1x107 AG3.0 hybridoma cells using the RNeasy plus kit (Qiagen) according to manufacturer's instructions and eluted in 30µl DEPC-treated H2O. CDNA was generated with the Cloned AMV cDNA synthesis kit (Invitrogen) using oligodT primers and a temperature of 50°C for 1h. Amplification of variable region fragments was performed with 1.25U Pfu DNA polymerase (Fermentas) in the supplied PCR buffer containing 2mM MgSO4, 0.2mM dNTPs and 25pmol of each primer per 50µl reaction. Forward and reverse primers from a mouse IgG library primer set (Progen) were used in all possible combinations for light (kappa forward: N-W, kappa reverse: X, lambda forward: Y, lambda reverse: Z) and heavy chain (forward: A-L, reverse: M) fragments. Amplification cycles were as follows: initial denaturation for 5 mins at 95°C, followed by 35 cycles of 30 sec/95°C, 30sec/55°C, 60sec/72°C and a final elongation of 10 mins at 72°C. Amplified PCR products of approximately 400kb were purified using a PCR purification kit (Fermentas) or, if more than one product was generated, separated on a 1.5% agarose gel and subsequently extracted using a gel extraction kit (Qiagen). Sequencing was performed on an ABI 3500DX sequencer. Sequences obtained were analysed using Vbase2 [193] to identify the complementarity determining regions of the heavy and light chain variable fragments.

2.2 Animals

In a previous study, twelve AGMs, (Chlorocebus aethiops) and twelve rhesus macaques (Macaca mulatta), housed in the animal facilities of the Paul-Ehrlich-Institute, had been immunised and experimentally infected with SIVagm or SIVmac, respectively. In addition to samples from this study, blood was taken from sixteen SIV-naïve or chronically SIV-infected AGMs and rhesus macaques. The animals were between 2-24 years old (both genders) and were seronegative for simian T-cell lymphotropic virus (STLV), SIVagm, SIVmac, simian retrovirus type D (SRV) and simian herpes B virus. All animal experiments were performed in accordance with institutional and state guidelines.

2.3 Immunisations

Animals had been immunised as part of a previous study (Siegismund et al., submitted). Briefly, microscopic (0.8-1.5µm) gold particles coated in DNA were delivered bioballistically to the dermis using a hand-held helium-pulsed gene gun (Biorad, Germany). Animals were divided into groups of four animals receiving either SIVagmGag-DNA, SIVmacGag-DNA or empty vector as control (table 2.3). Each monkey received sixteen non-overlapping 'shots' into freshly shaven abdominal skin at discharge pressures of 200 psi using 0.5 mg DNA-coated gold particles per shot. The 4µg DNA per inoculation consisted of 2µg pTH-SIVagm3GagCO or pTH17 SIVmac239GagCO or empty vector DNA co-immobilised with 2µg of the species specific GM-CSF DNA. Immunisations were performed at weeks 0, 3, 5 and 8. Three weeks after the last inoculation, the AGMs were infected with 0.25 ml pooled plasma from naturally infected AGMs and the rhesus macaques with 5000 MID50 SIVmac239 (Siegismund et al., submitted).

Table 2.3: Immunisation of AGMs and Rhs used in a previous study. Groups of 4 monkeys had either been immunised with pTH-SIVagmGag, pTH-SIVmacGag (all codon-optimised) or pTH-empty vector using Gene Gun with three boosts at weeks 3, 5 and 8 after initial vaccination. Animals were infected at week 11 post vaccination with SIVagm and SIVmac, respectively. Plasma samples were taken on a weekly basis post infection and stored at -80°C. Samples from weeks 0, 1, 2 and 3 post infection were used for the detection of cytokines.

AGM	pTH-SIVagmGagCO	pTH-SIVmacGagCO	pTH-empty	Rhesus	pTH-SIVagmGagCO	pTH-SIVmacGagCO	pTH-empty
AGM1	x			Rh13	x		
AGM2	x			Rh14	x		
AGM3	x			Rh15	x		
AGM4	x			Rh16	x		
AGM5		x		Rh17		x	
AGM6		x		Rh18		x	
AGM7		x		Rh19		x	
AGM8		x		Rh20		x	
AGM9			x	Rh21			x
AGM10			x	Rh22			x
AGM11			x	Rh23			x
AGM12			x	Rh24			x

2.4 Specimen collection

Seven to 21 mls of citrate blood were collected using vacutainer tubes (BD, Germany) from the femoral vein of each monkey one week before the first immunisation and at the time of each immunisation. Samples were also taken on the day of infection and at weeks 1, 2, 3, 4, 8,

13, 17, 20, 24, 33, 45, 51, 55 and 60 thereafter. PBMC and plasma were separated as described below, aliquoted and frozen at -80°C (plasma) or in liquid nitrogen (PBMCs) until use.

2.5 Determination of p27 concentration in AGM and rhesus plasma

Confirmation of the previous plasma virus load determination with realtime-PCR (Siegismund et al., submitted) was performed by detecting p27 protein in plasma samples taken at weeks 0, 1, 2, 3 and 4 post infection with SIVagm and SIVmac, respectively with a p27 detection ELISA kit (Zeptometrix) according to manufacturer's instructions.

2.6 Quantification of cytokines and chemokines in plasma

Plasma samples from week 0, 1, 2 and 3 p.i. of all animals were used for the measurement of biomarker levels. Seventeen cytokines/chemokines were measured using a high-sensitivity non-human primate cytokine Milliplex kit (Millipore, overnight protocol): GCSF, GM-CSF, IFN-gamma, IL-1beta, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-12, IL-15, IL-17, MIP-1alpha, MIP-1beta, sCD40L, TNF-alpha and IL-18. Each sample was assayed in duplicate, and cytokine standards supplied by the manufacturer were run on each plate. Data were acquired using a BioPlex 200 system and analysed using Bio-Plex Manager software, v4.1.1 (Bio-Rad). In addition, plasma levels of IFN-alpha and IFN-beta were detected with ELISA (Mabtech and PBL interferon source, respectively) according to manufacturer's intructions.

2.7 Isolation of PBMCs from AGM and rhesus blood samples

Citrate blood from naïve and chronically SIV-infected AGMs and rhesus macaques was separated on 15ml Histopaque (SIGMA) in Leucosept tubes at 1436xg for 25 minutes. Plasma and PBMCs were carefully transferred to separate secondary tubes and centrifuged at 3400xg for 10 minutes (plasma) to remove residual cells and cell debris or at 688xg for 10mins to pellet PBMC. If PBMC pellets were contaminated with red blood cells, they were resuspended in 0.86% ammonium chloride and incubated at 37°C for 20 mins. before washing twice with PBS. Resuspended PBMCs were subsequently counted using a particle count and size analyser (Z2, Coulter).

2.8 Sequencing of AGM and rhesus biomarker mRNAs

As the sequences of most AGM and rhesus biomarker genes were unknown, the development of AGM and rhesus biomarker realtime PCR assays required the sequencing of the respective mRNAs. Figure 2.10 shows the workflow for obtaining the required AGM biomarker sequences

2.9 Generation of AGM biomarker cDNA for amplification and sequencing

$1x10^7$ PBMCs from AGMs were stimulated for 17h with 10µg/ml LPS, 10µg/µl PHA or 5µg/µl ConA, respectively in RPMI medium (SIGMA), supplemented with 100U/ml Penicillin, 0,1mg/ml Streptomycin (PAA) and 10% fetal calf serum (SIGMA). Prior to RNA isolation, cells were washed once with PBS. RNA isolation from pelleted cells was performed using the RNeasy plus kit (Qiagen) according to the manufacturer's instructions. Subsequent cDNA synthesis was performed using the cloned AMV cDNA synthesis kit (Invitrogen) with oligodT primers and a temperature of 50°C for 1h. cDNA was stored at -20°C until further use.

2.10 Amplification of AGM biomarker cDNAs

Primer sets for the amplification of AGM biomarker cDNAs had been designed from the respective human and rhesus sequences using the Clone manager software. Tables 2.10a, b and c show the primers that yielded PCR products. A listing of more than one primer pair indicates that it was necessary to generate more than one product in order to obtain a sequence long enough to allow primer design for realtime PCR.1-2µg cDNA were used in each 50µl PCR reaction containing 0.5U Pfu polymerase (Fermentas) in the respective buffer (containing 20mM $MgSO_4$), 0.6µM forward and reverse primers and 0.5mM of each dNTP. Cycling conditions were as follows: Initial denaturation at 95°C for 10 mins, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 50°C for 30s and elongation at 72°C according to expected fragment length (<500bp= 30s, <1000bp= 1min, <2000bp= 2min). The final elongation step was at 72°C for 10 mins. 5µl of the PCR reactions were analysed on 1% agarose gels. If only one product was generated, the remaining 45µl of the PCR reaction were purified with a PCR purification kit (Qiagen) according to manufacturer's instructions for sequencing.

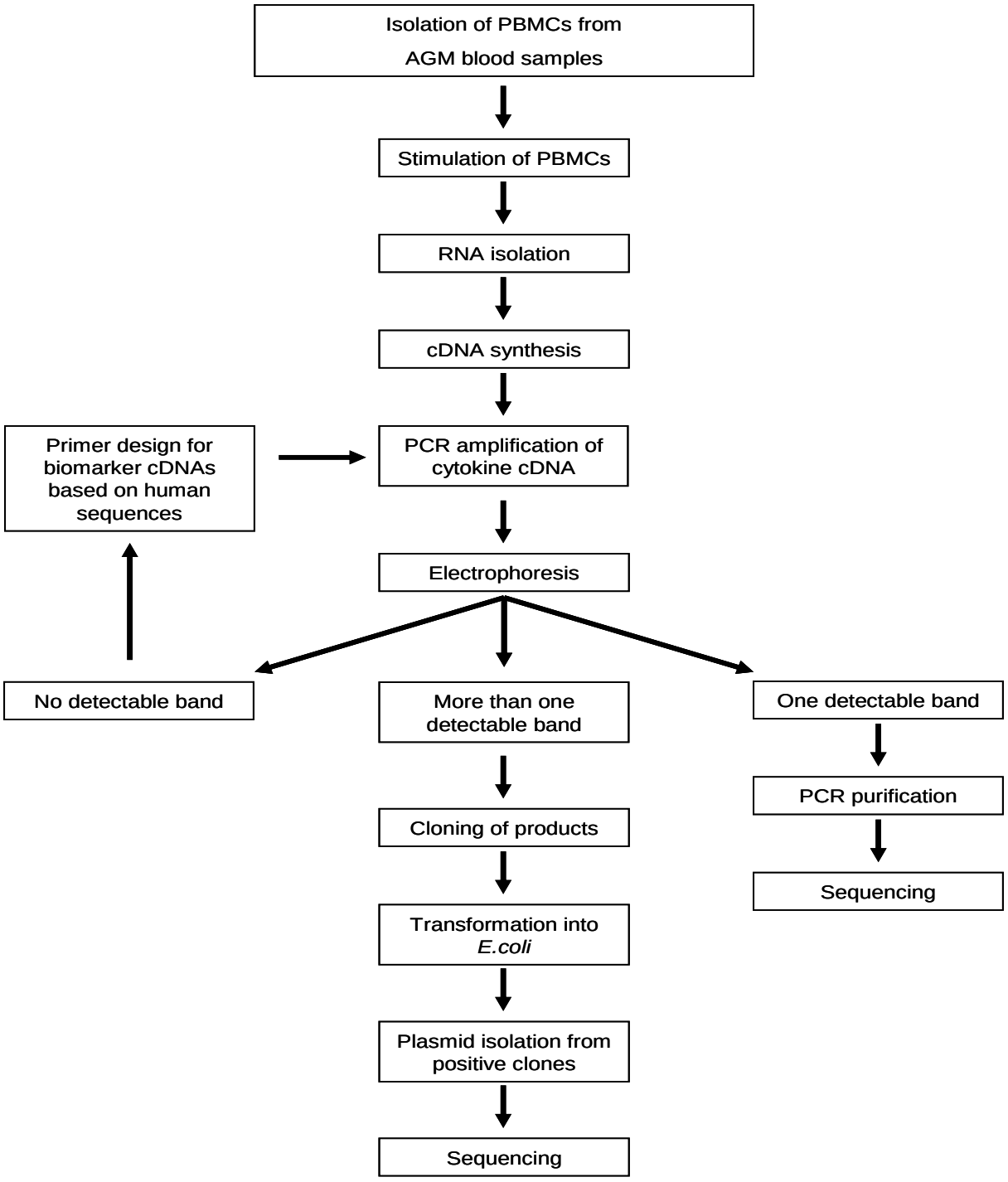

Figure 2.10: Workflow for biomarker sequencing. Processes involved starting from the isolation of AGM PBMCs to the sequencing of biomarker cDNAs. AGM PBMCs were isolated, stimulated with LPS, PHA and ConA, respectively, followed by RNA isolation and cDNA synthesis. Primer sets for the amplification of AGM biomarker cDNAs had been designed from the respective human and Rh sequences. PCR reactions were analysed on 1% agarose gels. If only one product was generated, the PCR reactions were purified for sequencing. If the amplification of AGM biomarker cDNAs resulted in more than one band after agarose gel electrophoresis, bands were cut out and DNA was extracted. Subsequently, PCR products were cloned into a Topo-Blunt vector (Invitrogen) and electroporated into Top10 E.coli. Transformed bacteria were plated out on LB-ampicillin agarose plates and incubated at 37°C overnight. Following overnight culture of bacterial suspensions, plasmids were isolated and sequenced. BigDye Premix (Perkin-Elmer) was used for each sequencing PCR according to manufacturer's instructions. Purification via gel filtration and read-out of the sequencing reaction was performed by the personnel of the sequencing core facility at the Robert Koch-Institute and data was analysed using the BioEdit software.

Table 2.10a: Primers designed for the amplification of AGM biomarker cDNAs

Marker	Primer orientation	Primer sequence 5' - 3'	Primer reference or Genbank accession numbers used for primer design
FoxP3	fwd 1	CCTCYTCTTCYTCCTTGAAC	NM_001032918.1; NM_014009.3; NM_001114377.1
	rev 1	TTCTTGCGGAACTCCARC	
	fwd 2	TGCTGGCAAATGGTGTCTG	
	rev 2	AGCGTGGCRTAGGTGAAAG	
IFN gamma	fwd	TGCTCTAGATGAAATATACAAGTTATATCTTGGCT	[194]
	rev	GGAATTCACTGGGATGCTCTTCGACCTCGA	
IFN beta	fwd	TCCTGTTGTGCTTCTCCACTAC	EF064725.1; NM_001135795.1
	rev	AGGCACAGGCTAGGAGATCTTC	
IL-1 alpha	fwd	ATGGCCAAAGTTCCAGAC	BC013142.2; NM_001042757.1
	rev	GGATGGGCAACTGATATG	
IL-10	fwd	TGCTCTAGAAGGCATGCACAGCTCAGC	[194]
	rev	TCTCAAGGGGCTGGGTCAGCTATCCCA	
IL-12 alpha	fwd	GCCCAGAGCAAGATGTGTCA	[194]
	rev	TGGGTCTATTCCGTTGTGTCTTTA	
IL-12 beta	fwd	CTGTTTCAGGGCCATTGGAC	NM_002187.2; NM_001044725.1
	rev	AGTTCAGCCTCAGAATGC	
IL-13	fwd	CGGTCATTGCTCTCACTTG	NM_002188.2; NM_001032929.1
	rev	GTACGAGCAGGTCCTTTAC	
IL-15	fwd	GACTCGAGAAGCTTAAGGATTTACCGTGGCTTTGAG	[194]
	rev	TCGAATTCTAAGCAGCAGAGTGATGTTCGTT	
IL-18	fwd	GGCTGCTGAACCAGCAGAAGAC	NM_001562.2; NM_001032834.1
	rev	GTCCTGGGACACTTCTC	
IL-2	fwd	AGTAACCTCAACTCCTGCCACAATG	NM_001047130.1; NM_001047130.1
	rev	TGGTTGCTGTCTCATCAG	

Table 2.10b: Primers designed for the amplification of AGM biomarker cDNAs

Marker	Primer orientation	Primer sequence 5' - 3'	Primer reference or Genbank accession numbers used for primer design
IL-22	fwd 1	ACTTCCAGCAGCCCTATATC	NM_020525.4; NM_001194724.1
	rev 1	GAGTTTGGCTTCCCATCTTC	
	fwd 2	GAAATCTGTGAGCYCTTTCC	
	rev 2	GAGTTTGGCTTCCCATCTTC	
IL-4	fwd	AAAAGCCAGCAGCAGCCCCAAGC	GeneBank NM_000589.2; NM_172348.1; L26027.1
	rev	AGGACAGGAATTCAAGCCCGCCAGG	
IL-5	fwd	GCCAAAGGCAAACGCAGAAC	NM_000879.2; NM_001047133.1
	rev	GCTTTCTGGCAAAGTGTC	
IL-6	fwd	ATGAACTCCTTCTCCACAAG	[194]
	rev	CGGAATTCTACATTTGCCGAAGAGCCCTCAG	
IL-7	fwd 1	CGCAGACCATGTTCCATGTTTC	NM_000880.2; NM_001032846.1
	rev 1	TGGAGGATGCAGCTAAAGTTCG	
	fwd 2	GTTGCGGTCATCATGACTAC	
	rev 2	CTTGGAGGATGCAGCTAAAG	
IL-8	fwd	CAAGAGCCAGGAAGAAACCAC	[194]
	rev	GGCATCTTCACTGATTCTTGG	
IP-10	fwd	GACATATTCTGAGCCTACAGCAGAG	BC010954.1; NM_001032892.1
	rev	GTATGTAGGTAGCCACTGAAAG	
IRF-7	fwd 1	TAGCAGCAGRGGAGGTGRC	EU204916; EU204917; NM_001572
	rev 1	CAGCAGTTCCTCCGTGTAG	
	fwd 2	GATCAGCAGCGGCTGCTATGAG	
	rev 2	GTGCGGCCCTTGTACATGATGG	
	fwd 3	AGAGGGACAAGAGGGTCTTC	
	rev 3	TGGTGCTGGACAGACAGAG	
	rev 4	GCTCCAGCTCCATGAGSAARC	

Table 2.10c: Primers designed for the amplification of AGM biomarker cDNAs

Marker	Primer orientation	Primer sequence 5' - 3'	Primer reference or Genbank accession numbers used for primer design
MCP-1	fwd 1	ATAGCAGCCACCTTCATTCC	NM_002982.3; NM_001032821.1
	rev 1	GGGTAGAACTGTGGTTCAAGAG	
	fwd 2	TCATAGCAGCCACCTTCATTCC	
	rev 2	GGGTAGAACTGTGGTTCAAGAG	
MIP-1 alpha	fwd 1	TCTCTGCAACCRGWTCTC	NM_002983.2; NM_001034200.1
	rev 1	TGGACCCACTCCTTACTG	
	fwd 2	TCTCTGCAACCGGATCTC	
	rev 2	TCAGGCACTCAGCTCTAGG	
MIP-1 beta	fwd 1	GCCACCAATACCATGAAG	NM_002984.2; NM_001032873.1
	rev 1	GCAACAGCAGAGAAACAG	
	fwd 2	CCTCTTTGCCACCAATAC	
	rev 2	GCAACAGCAGAGAAACAG	
PD-1	fwd 1	TGCTCCAGGCATGCAGATCC	NM_005018.2; NM_001114358.1
	rev 1	YRGAACACCAGTGGCCAAGG	
	fwd 2	TGGCGGCCAGGATGGTTCTTAG	
	rev 2	TGYRGAACACCAGTGGCCAAGG	
PDL-1	fwd	CTGCAGGGCATTCCAGAAAG	NM_014143.3; EF444816.1
	rev	CCAATGCTGGATTACGTCTC	
PDL-2	fwd	GTCCCAGCTAGAAAGAATCC	GeneBank NM_025239.3; NM_001083599.1
	rev	GCTTGCTTTAGGCCCACCTATG	

Table 2.10d: Primers designed for the amplification of AGM biomarker cDNAs

Marker	Primer orientation	Primer sequence 5' - 3'	Primer reference or Genbank accession numbers used for primer design
RAN-TES	fwd 1	GTCATCCTCRTTGCTACWG	NM_002985.2; NM_001032850.1
	rev 1	CCCGAACCCATTTCTTCTC	
	fwd 2	TCGCTGTCATCCTCRTTGCTAC	
	rev 2	ACTCCCGAACCCATTTCTTCTC	
TNF alpha	fwd	ATGAGCACTGAAAGCATGATC	[194]
	rev	TCACAGGGCAATGATCCCAAAGTAGACCTGC	
VEGF	fwd 1	GCAGCTACTGCCATCCAATCG	NM_001171623.1; NM_001171624.1; NM_001171625.1; NM_001171626.1; NM_001171627.1; NM_001171628.1; NM_001171629.1; AF339737.1
	rev 1	CTATGTGCTGGCCTTGGTGAG	
	fwd 2	TACTGCCATCCAATCGAGAC	
	rev 2	TGTGCTGTAGGAAGCTCATC	

2.11 Cloning of AGM and rhesus biomarker genes

If the amplification of AGM biomarker cDNAs resulted in more than one band after agarose gel electrophoresis, bands were cut out and DNA was extracted using a gel extraction kit (Qiagen) according to manufacturer's protocol. Subsequently, PCR products were cloned into a Topo-Blunt vector (Invitrogen) according to the manufacturer's protocol and electroporated into Top10 *E.coli.* Transformed bacteria were plated out on LB-ampicillin agarose plates and incubated at 37°C overnight.

2.12 Detection of positive bacterial clones

For each transformed insert, eight grown colonies were screened by colony PCR. Briefly, each bacterial colony was resuspended in 200µl LB-amp medium and 1µl of the suspension was used in a 25µl PCR reaction containing 0.5U Taq polymerase (Fermentas) in the respective buffer, 4ng of each M13 forward and reverse primers (supplied with the Topo-Blunt cloning kit, Invitrogen), 0.5mM of each dNTP and 15mM $MgCl_2$. Cycling conditions

were as follows: Initial denaturation at 95°C for 15 mins, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and elongation at 72°C according to expected fragment length (<500bp= 30s, <1000bp= 1min, <2000bp= 2min). The final elongation step was at 72°C for 10 mins. PCR reactions were analysed on 1% agarose gels. For each biomarker, two positive clones were inoculated into 5ml LB-amp and shaken at 37°C and 225rpm overnight.

2.13 Plasmid isolation

Following overnight culture, bacterial suspensions were centrifuged at 3400xg for 20 mins and the supernatant discarded. Plasmids were isolated from the bacterial pellets using a Plasmid Miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was eluted with 30µl H_2O.

2.14 Quantification of RNA and DNA

RNA/DNA concentrations of samples were determined using a NanoDrop ND-1000 (Peqlab) spectrometer at 260nm. Samples were considered free of protein impurities if the extinction coefficient ratio (260/280nm) was 1.8 or higher.

2.15 Sequencing of AGM and rhesus biomarker cDNAs

BigDye Premix (Perkin-Elmer) was used for each sequencing PCR according to manufacturer's instructions. Briefly, 10-20ng of purified PCR product or 150-300ng plasmid DNA were used in each sequencing reaction containing 10pmol gene-specific sequencing primer (the same forward or reverse primer previously used for PCR amplification), 1µl 5x ABI buffer (Perkin Elmer) made up to 10µl with H_2O. Cycler conditions for sequencing reactions were as follows: Initial denaturation at 96°C for 2 mins, followed by 25 cycles of 96°C for 10 sec., 55°C for 5 sec and 60°C for 4 mins. Sequencing reactions were stored at -20°C until further use. Purification via gel filtration and read-out of the sequencing reaction was performed by the personnel of the sequencing core facility at the Robert Koch-Institute and data was analysed using the BioEdit software. The sequences obtained for AGM

biomarkers are listed in the appendix. For the following biomarkers, AGM cDNA sequences were already available in Genbank:

GM-CSF: DQ845250.1
IFN alpha: FJ194491.1
IL-16: AF017106.1
IL-1Ra: AY172101.1

2.16 Primer design for biomarker real-time PCRs

The newly acquired AGM biomarker cDNA sequences were aligned with their respective rhesus cDNA and human genomic DNA sequences to identify likely splicing sites. Primers were designed to be compatible with both AGM and rhesus sequences to facilitate their use for both species. In addition, the primers were matched to different (predicted) exons with intron sequences with enough separation to make amplification of genomic DNA unlikely (figure 2.16). The lengths of all amplified realtime-PCR products were 140-288bp. The primer pairs designed for realtime-PCR are listed in tables 2.16a and 2.16b. The primers used for the quantification of GapdH have been described previously [195].

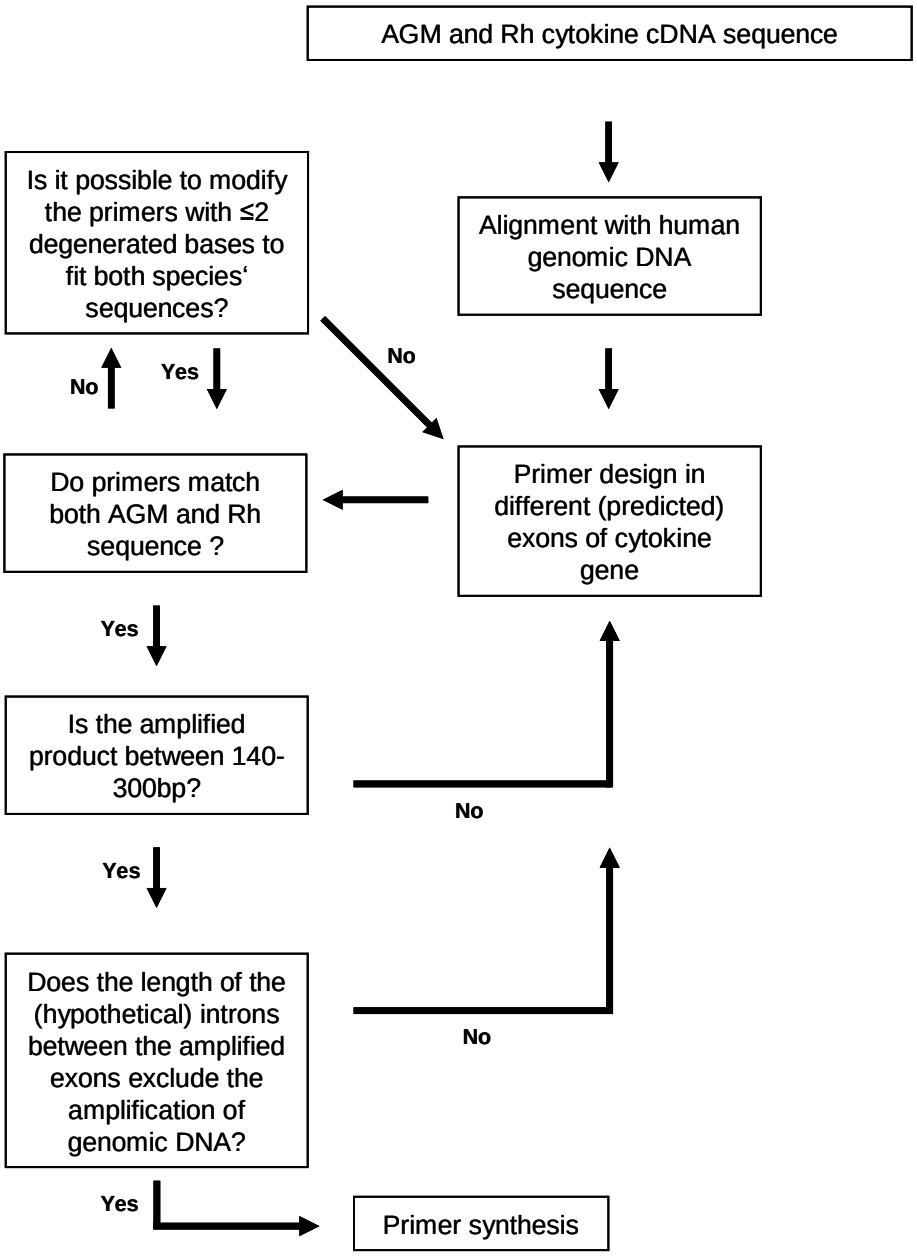

Figure 2.16: Workflow primer design, showing the criteria for the design of primers used to quantify biomarker mRNA expression levels in AGM and Rh PBMCs. The newly acquired AGM biomarker cDNA sequences were aligned with their respective Rh cDNA and human genomic DNA sequences to identify likely splicing sites. Primers were designed to be compatible with both AGM and Rh sequences to facilitate their use for both species. In addition, the primers were matched to different (predicted) exons with intron sequences with enough separation to make amplification of genomic DNA unlikely. The lengths of all amplified realtime-PCR products were 140-288bp.

Table 2.16a: Primers Designed for realtime PCR
* Unspliced gene, needs -RT control for each PCR

Marker	Primer orientation	Sequence 5' → 3'	Location in AGM cDNA sequence (see appendix)	Located in predicted exon	Length of PCR product (bp)	Length of predicted introns (bp)
FoxP3	fwd	GAGAAGCTGAGTGCTATG	586	7	225	2073
	rev	GAAGGAACTCTGGGAATG	811	8-9		
GM-CSF	fwd	AACCGTAGAAGTCGTCTC	165	2	239	1494
	rev	GGGATGACAAGCAGAAAG	404	4		
IFN alpha	fwd	AGGAGTTTGGCAACCAGTTC	259	1	244	0*
	rev	GTATTTCCTCACRGCCAGGATG	503	1		
IFN beta	fwd	CTAGCACTGGCTGGAATG	211	1	202	0*
	rev	GGCACAGTGACTGTACTC	413	1		
IFN gamma	fwd	CAGGTGATCCAGATGTAG	120	2	271	1411
	rev	GCGTTGGACATTCRAGTC	391	4		
IL-1 alpha	fwd	TCACGGCTGCTRCAATAC	338	2	174	4101
	rev	ATCTCAGGCATCTCCTTC	512	4		
IL-10	fwd	TTCGAGACCTCCGAGATG	129	1	203	1149
	rev	CATGCTCCTTGATGTCTG	332	3		
IL-12 alpha	fwd	ACCCTTGTACTTCTGAAGAG	107	2	222	485
	rev	ACYTGGTACATCTTCAAGTC	329	5		
IL-12 beta	fwd	AGGAGAGGCTGCCCATTGAG	385	3	232	3906
	rev	CTTGCCCTGGACCTGRATGC	617	4		
IL-13	fwd	GGAGCTGGTCAACATCAC	84	1	167	1307
	rev	CAGAATCCGTTCAGCATCC	251	3		
IL-15	fwd	TTTCCATCCAGTGCTACTTG	59	2	288	9150
	rev	ATATCTGTATCTCCGGACTC	347	5		
IL-16	fwd	CGGAAGCCAAGGAAGATG	1175	3	200	2035
	rev	CCAAGCTGAACCCAAGAC	1375	4		
IL-18	fwd	ACTGTAGCCATCTCTGTG	2	1	99	4784
	rev	TCCTTGATGTTATCAGGAG	101	2		
IL-1Ra	fwd	CTGCCGTTCATCTCATAC	397	3	218	3585
	rev	GTAATAGCCCGCATCTTC	615	4		
IL-2	fwd	GTCACAAACAGTGCACCTAC	71	1	214	2378
	rev	TCCTCCAGAGGTTTGAGTTC	285	3		
IL-22	fwd	CAGCCCTATATCACCAAC	125	1	279	1470
	rev	GGATATGCAGGTCATCAC	404	4		
IL-4	fwd	GGACACAACTGCCATATCG	11	1	233	5206
	rev	CTTGTGCCTGTGRAACTG	244	3		

Table 2.16b: Primers Designed for realtime PCR
* Unspliced gene, needs -RT control for each PCR
** Different splice variants, two different products are generated

Marker	Primer orientation	Sequence 5' → 3'	Location in AGM cDNA sequence (see appendix)	Located in predicted exon	Length of PCR product (bp)	Length of predicted introns (bp)
IL-5	fwd	TGGCACTGCTTTCTACTC	102	1	257	1261
	rev	GCAGGTAGTCTAGGAATTGG	359	4		
IL-6	fwd	CAGAGCTGTGCARATGAG	5	1	102	1744
	rev	GCTGGCATTTGTGGTTG	107	2		
IL-7	fwd	GTTGCCAGTAGCATCATC	43	1	161/308	61476**
	rev	TTAACCTTGCCGGTGCAG	204/351	4		
IL-8	fwd	CAAGAGCCAGGAAGAAAC	1	1	228	818
	rev	TGTGGTCCACTCTCAATC	229	2		
IP-10	fwd	CTGATTTGCTGCCTTGTC	50	1	227	786
	rev	GATGGCCTTAGATTCTGG	277	3		
IRF-7	fwd	CACATGGGCAGTAGAAGC	649	6	184	188
	rev	GGCCCTTGTACATGATGG	833	7		
MCP-1	fwd	CATAGCAGCCACCTTCAG	21	1	185	1177
	rev	ATCTCCTTGGCCACAATG	206	3		
MIP-1 alpha	fwd	GCAACATTTGCTGCTGACAC	2	1-2	143	419
	rev	CCGGCCTCTCTTGGTTAG	145	3		
MIP-1 beta	fwd	GCTAGYAGCTGCCTTCTG	30	1	315	1126
	rev	AGGAGATGCCTCTCATRG	345	3		
PD-1	fwd	CTGGTGCTGCTAGTCTG	465	2	213	863
	rev	CRATGGTGGCGTACTCC	678	4		
PDL-1	fwd	AGCTCTCCCTGGGAAATG	236	2	246	5412
	rev	TGATGGTCACTGCTTGTC	482	3		
PDL-2	fwd	CTCCAGCAAAGTGGCTCTTTC	240	2	199	12142
	rev	AACTGCAGCTTCACCAGAC	439	3		
RANTES	fwd	CCTGCTGCTTTGCCTACATTG	95	1	123	0*
	rev	CACACTTGGCGATTCTTTCGG	218	1		
TNF alpha	fwd	CTGTCTGCTGCACTTTGG	114	1	275	1091
	rev	AAGAGGACCTGGGAGTAG	389	4		
VEGF	fwd	CAGGAGTACCCTGATGAGATTG	167	1	140	0*
	rev	GATCCGCATAATCTGCATGG	307	1		

2.17 Generation of positive control DNA templates for realtime-PCR

DNA control templates for biomarker realtime-PCRs were generated using the designed realtime-PCR primer set for each biomarker in a 50µl PCR setup containing 1-2µg cDNA from previously stimulated AGM and rhesus PBMCs, 1U *Taq* polymerase (Fermentas) in the respective buffer, 0.6µM forward and reverse primers, 0.5mM of each dNTP and 1mM $MgCl_2$. Cycling conditions were as follows: Initial denaturation at 95°C for 10 mins, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 56°C for 30s and elongation for 30s at 72°C. The final elongation step was at 72°C for 10 mins. The PCR reactions were analysed on 1% agarose gels, bands were cut out and DNA was extracted using a gel extraction kit (Qiagen) according to the manufacturer's protocol.

2.18 Testing of realtime-PCR assay efficiency with AGM and rhesus cDNAs

The cDNAs generated from stimulated AGM and rhesus PBMCs were diluted in three ten-fold steps. Each diluted sample was run in triplicate in a Stratagene MX3005P light cycler using a 25µl realtime PCR setup containing 0.1µl diluted template, 1U hot start *Taq* polymerase in the respective buffer (HS1, Segenetic) with an additional 3mM $MgCl_2$, 0.5µl of a 50x concentrated Eva Green fluorescent DNA stain (Jena Bioscience), 80nM of each forward and reverse primer and 0.63mM dNTPs. Cycling conditions were optimised to be as follows: Initial denaturation at 95°C for 10mins, followed by 50 cycles of denaturation at 95°C for 10s, annealing at 56°C for 30s and elongation at 72°C for 15s, followed by one dissociation curve cycle of denaturation at 95°C for 1min, annealing at 55°C for 30s and melting until reaching a final temperature of 95°C for 30s. The means and standard deviations were calculated from the ct values of each triplicate sample (using the same threshold for all the samples in the same experiment on the same plate). Using Excel, mean ct values were then plotted against the dilutions and the correlation coefficient R^2 (i.e. the efficiency of the PCR) was calculated. This was done to ensure that the PCR efficiencies for AGM and rhesus cDNA were within the same range of ct values.

2.19 Determination of biomarker expression levels in PBMCs of chronically SIV-infected AGMs and rhesus macaques using realtime RT-PCR

The method, described above, initially used for synthesising cDNA from cell pellet RNA necessitated a 555-fold dilution of the original RNA in the realtime PCR setup as higher amounts of the cDNA synthesis reaction (containing EDTA) completely inhibited the assay. This prevented the measurement of biomarkers expressed at low levels. In addition, the RNA in PBMC pellets ($5x10^5$ cells) from AGMs and rhesus macaques of the earlier SIV*gag*DNA immunisation study were found to be partially degraded.

Aliquots of $1x10^7$ PBMCs were therefore isolated within one hour of blood sampling from eight chronically SIV-infected AGMs and rhesus macaques and directly lysed (for later RNA isolation) as described above. RNA from disrupted cells was stabilised in the lysis buffer provided in the RNA isolation kit (RNeasy plus, Qiagen), shipped to the RKI on dry ice and stored at -80°C until further use. Biomarker expression levels were determined using the primers described above and a commercial one-step SYBR Green RT-PCR master mix (Quantitect, Qiagen) that allowed the direct use of RNA in place of cDNA.

80nM biomarker forward and reverse primers and 3µl RNA template (isolated from PBMC lysates using the Qiagen RNeasy plus kit directly before running the RT-PCR) were added to the ready-to-use RT-PCR master mix, giving a total reaction volume of 25µl. Samples were run in duplicate with cycling conditions as follows:
RT reaction at 50°C for 30mins, followed by an initial denaturation at 95°C for 15mins and a subsequent 50 cycles of denaturation at 94°C for 10s, annealing at 56°C for 30s and elongation at 72°C for 30s, followed by one dissociation curve cycle of denaturation at 95°C for 1min, annealing at 55°C for 30s and melting until reaching a final temperature of 95°C for 30s. From each biomarker measurement, the copy number/GAPDH ratio was calculated as follows:

Assuming a ct of 40 to be equivalent to one copy, the number of copies in each reaction was calculated as 2^(40-mean CT) . The ratio of biomarker copies/ GAPDH copies was then calculated for each biomarker in each animal.

2.20 Quantification of pDCs in PBMCs with FACS

Flow cytometric analysis was performed with an LSRII flow cytometer on freshly isolated cells from SIV negative AGMs and rhesus macaques. Cells were stained as previously described [196] with the following monoclonal antibodies: fluorescein isothiocyanate (FITC)–labeled anti-Lineage (Lin) panel: anti-CD3 (FN18; Biosource) / CD14 (TüK4; Miltenyi) / CD16 (3G8;BD) / CD20 (2H7;BD), peridin chlorophyll (PerCP)–labeled anti-HLA-DR (L243;BD) and phycoerythrin (PE)–labeled anti-CD123 (7G3; BD). PDCs were defined as Lin- HLA-DR+ CD123+ (Fig. 2.20).

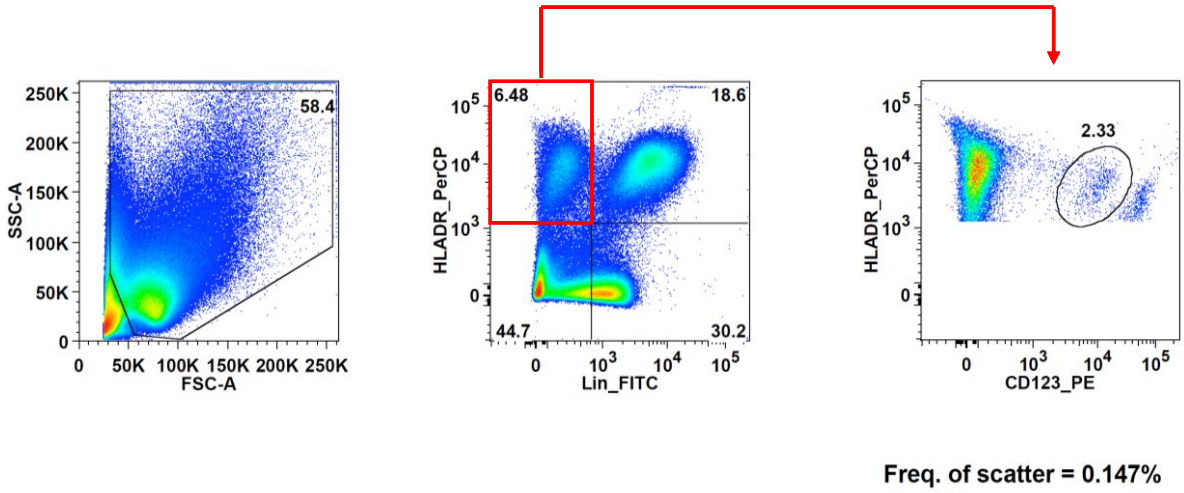

Figure 2.20: Gating strategy for the quantification of pDCs in PBMCs of AGMs and Rhs with FACS. Flow cytometric analysis was performed on freshly isolated cells from SIV negative AGMs and Rhs. Cells were stained with the following monoclonal antibodies: FITC–labeled anti-Lineage (Lin) panel: anti-CD3 (FN18; Biosource) / CD14 (TüK4; Miltenyi) / CD16 (3G8;BD) / CD20 (2H7;BD), anti-HLA-DR PerCP (L243;BD) and anti-CD123 PE (7G3; BD). PDCs were defined as Lin^- $HLA\text{-}DR^+$ $CD123^+$. Freq. of scatter: frequency of pDCs in PBMCs

2.21 Quantification of $CD20^+$ B-cells in PBMCs with FACS

Flow cytometric analysis was performed with an LSRII flow cytometer on freshly isolated cells from SIV negative AGMs and rhesus macaques. Cells were stained as described previously [196] with the following monoclonal antibodies: fluorescein isothiocyanate (FITC)–labeled anti-CD20 (2H7;BD) and peridin chlorophyll (PerCP)–labeled anti-CD3 (FN18;Biosource).$CD20^+$ B-cells were defined as $CD20^+$ $CD3^-$ (Fig. 2.21).

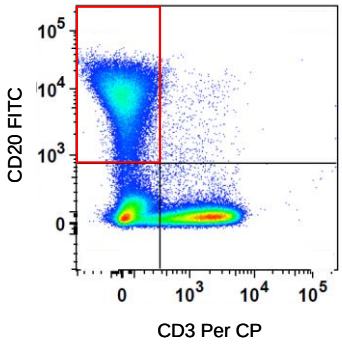

Figure 2.21: Gating for the quantification of CD20+ B-cells in PBMCs of AGMs and Rhs with FACS. Flow cytometric analysis was performed with an LSRII flow cytometer on freshly isolated cells from SIV negative AGMs and Rhs. Cells were stained anti-CD20 FITC (2H7;BD) anti-CD3 PerCP (FN18;Biosource). $CD20^+$ B-cells were defined as $CD20^+$ $CD3^-$.

2.22 Interferon alpha ELISPOT

Single cells secreting IFN-alpha were detected using a human IFN-alpha ELISPOT kit (Mabtech) according to the manufacturer's instructions. PBMCs from SIV negative AGMs and rhesus macaques were incubated in complete RPMI (Invitrogen) at 400,000 cells per well in duplicate wells with 1 mM R-848 (3M Pharmaceuticals), 6 mg ml–1 CpG C2395 or CpG A2336 or 500 ng ml–1 (total protein) aldrithiol-2–inactivated SIVmac239, aldrithiol-2–inactivated SIVagm, aldrithiol-2–inactivated HIV-1ADA or SUPT1 cell–derived microvesicles as a control (the latter four were kindly provided by Prof. J. Lifson, see acknowledgements) for 17 h at 37°C. For some stimulations, inhibitors of TLR7 were added: 5.6 mM IRS661, an oligodeoxynucleotide control (Invitrogen), or 20 mM chloroquine (Sigma). Quantity, size and darkness of spots were measured using an ELISPOT reader (Perkin Elmer).

2.23 Total IgG ELISA

In a preliminary experiment, plasma samples taken at different time points before and after infection of AGMs and rhesus macaques with SIVagm and SIVmac, respectively, were diluted in PBS in a series of 16 two-fold steps from 1:100-1:3.276.800 and left to dry overnight. Plates were then blocked with PMT (PBS, 0.05% Tween 20, 2% milk powder) for 1h at 37°C, followed by 6 washes with PBST (PBS with 0.05% Tween 20). Fifty µl of a peroxidise-labelled anti-human IgG antibody (SIGMA) diluted 1:1000 in PMT were then added to each well and the plates incubated for 1h at 37°C. Following a further six washes with PBST, 50µl substrate (100ml citrate buffer pH 5.0 with 60mg OPD (SIGMA) and 40µl 30% H_2O_2) were added and colour developed for 5-10min before addition of 20µl H2SO4 to

stop the reactioon. The OD 492nm was then measured using an ELISA reader (Sunrise, Tecan).

In a second experiment, plasma samples taken before and at weeks 2, 3, 4, 12, 20 and one year after infection of AGMs and rhesus macaques were diluted 1:10.000 and dried on ELISA plates before detecting IgG as described above.

3 Results

3.1 Sequencing of the AG 3.0 antigen binding site.

The initial goal of this project was the cloning of a recombinant anti-Gag antibody gene into an adeno-associated virus (AAV) vector. This was to be used for "gene therapy" in AGMs to artificially produce sustained high anti-Gag antibody titers prior to and following SIVagm infection in order to test the hypothesis that the lack of such antibodies played a major role in protection from disease in the natural hosts. The (murine) monoclonal antibody AG3.0 is specific for SIVagm p27 and cross-reacts with the Gag protein of members of the HIV-2 lineage (HIV-2, SIVmac), HIV-1 lineage, and SIVrcm (Sanders-Beer et al., submitted). It therefore represented an ideal candidate for the generation of a recombinant anti-Gag antibody. The intention was to artificially "fuse" the cDNA encoding the (murine) AG3.0 Fab fragment to an Fc-region derived from AGMs. In order to do so, the cDNA sequence for the AG3.0 complementarity determining regions (CDRs) of the Fab fragment had first to be determined.

CDNAs generated from RNA coding for the variable fragments of the AG 3.0 heavy and light chains resulted in the generation of 6 and 3 products for the heavy and light chain, respectively (figure 3.1a).

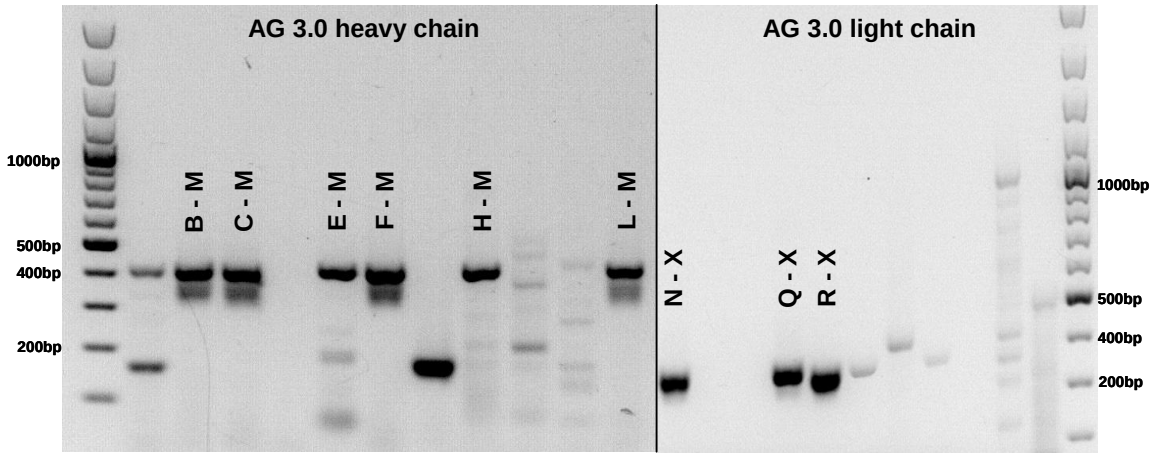

Figure 3.1a: PCR amplification of the AG3.0 hybridoma antibody cDNA.Forward and reverse primers from a mouse IgG library primer set (Progen) were used in all possible combinations for light (kappa forward: N-W, kappa reverse: X) and heavy chain (forward: A-L, reverse: M) fragments. Amplified PCR products were separated on a 1.5% agarose gel. Primer combinations that resulted in products used for sequencing are indicated. DNA marker: Fermentas Gene Ruler^{TM} 100bp DNA ladder.

The sequenced products were analysed using the Vbase2 application [193] to identify the conserved framework regions (FR1-FR4) and variable complementarity determining regions (CDR1-CDR3) according to the IMGT nomenclature. The three variable heavy chain regions were found to consist of 8, 8 and 16 amino acids for CDR1, CDR2 and CDR3 respectively whereas the light chain had corresponding CDR lengths of 11, 3, and 9 (figure 3.1b).

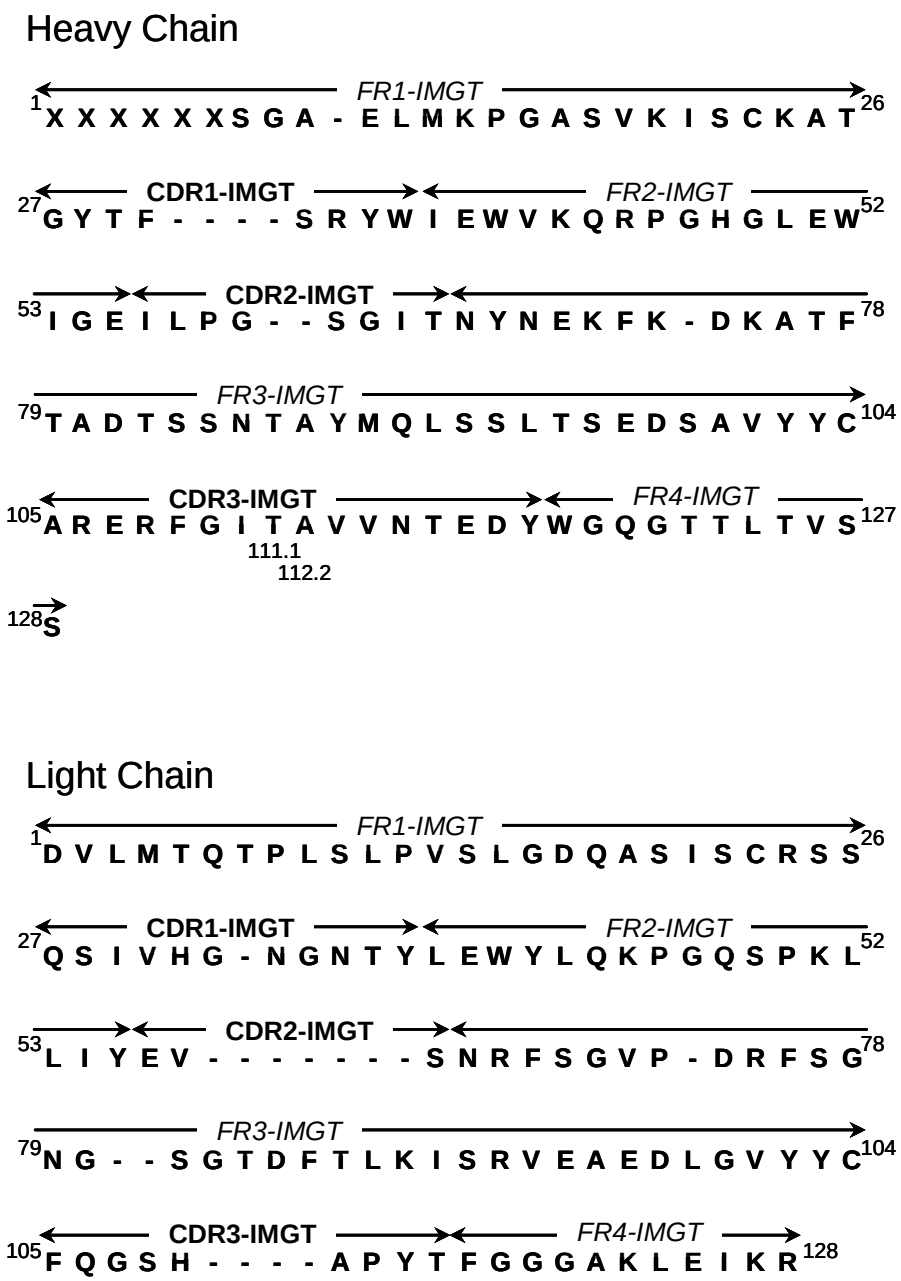

Figure 3.1b: Amino acid sequences of the CDRs of the AG 3.0 monoclonal antibody. The four conserved framework regions (FR1-FR4) and the three hypervariable complementarity determining regions (CDR1-CDR3) are displayed according to the IMGT nomenclature. Unoccupied amino acid positions are marked with a dash (-). X = positions in the conserved FR1 region of the heavy chain at which the identity of the amino acid could not be unequivocally determined from the sequence.

However, after beginning this project, data suggesting that the secondary antibody response to the SIV Gag protein strongly depends on T-cell help, whereas the response to (trimeric) SIV Env is T-cell independent was made public [197]. It is widely acknowledged that infected natural hosts for SIV do not exhibit T-cell hyperactivation [162]. A lack of T-cell help in SIVagm infected AGMs would therefore better explain the lack of (T-dependent) Gag-specific B-cell responses but formation of (T-independent) Env-specific antibodies in these animals. The absence of anti-Gag antibodies in SIVagm-infected AGMs might therefore be simply a bystander effect rather than the reason for the lack of disease progression in these animals. In order to identify candidate mechanisms leading to such a (systemic) lack of B-cell activation in AGMs, it was therefore decided to take a step back and obtain a broader overview of the immune activating and/or down-regulating processes in AGMs and rhesus macaques during SIV infection. The samples to be examined were derived from AGMs and rhesus macaques used in a previous experiment involving immunisation with SIV *gag* DNA before infection. A necessary first step was therefore to confirm that the AGMs experienced plasma virus loads at least as high as the macaques to rule out the possibility that, in this study at least, the lack of Gag-specific antibodies was simply due to lower quantities of antigen.

3.2 Plasma virus load in acutely SIV-infected AGMs and rhesus macaques of the immunisation study

Plasma virus loads in AGMs and macaques infected with SIVagm or SIVmac peaked at one to three weeks p.i. (Siegismund et al., submitted). The median loads in AGMs and rhesus macaques, as measured by real time RT-PCR were $9.7x10^5$ and $2.9x10^5$ copies/ml, respectively, and median steady-state viremia set points were $4x10^3$ copies/ml in AGMs and $1.6x10^3$ copies/ml in rhesus macaques.

SIVagm-infected AGMs therefore appeared to have virus loads at least as high, if not higher, than those in SIVmac infected macaques. However, it seemed possible that differences in the PCR setups for the two viruses might give misleading data. Therefore, as an independent verification of the relative plasma virus loads, levels of plasma p27 during the acute phase of SIV infection were measured using an antigen-capture ELISA designed for detection of SIVmac Gag. This indeed revealed higher levels of circulating p27 in AGMs during the first three weeks of infection compared to those in rhesus macaques, which in most cases had levels below the detection limit of the assay (figure 3.2).

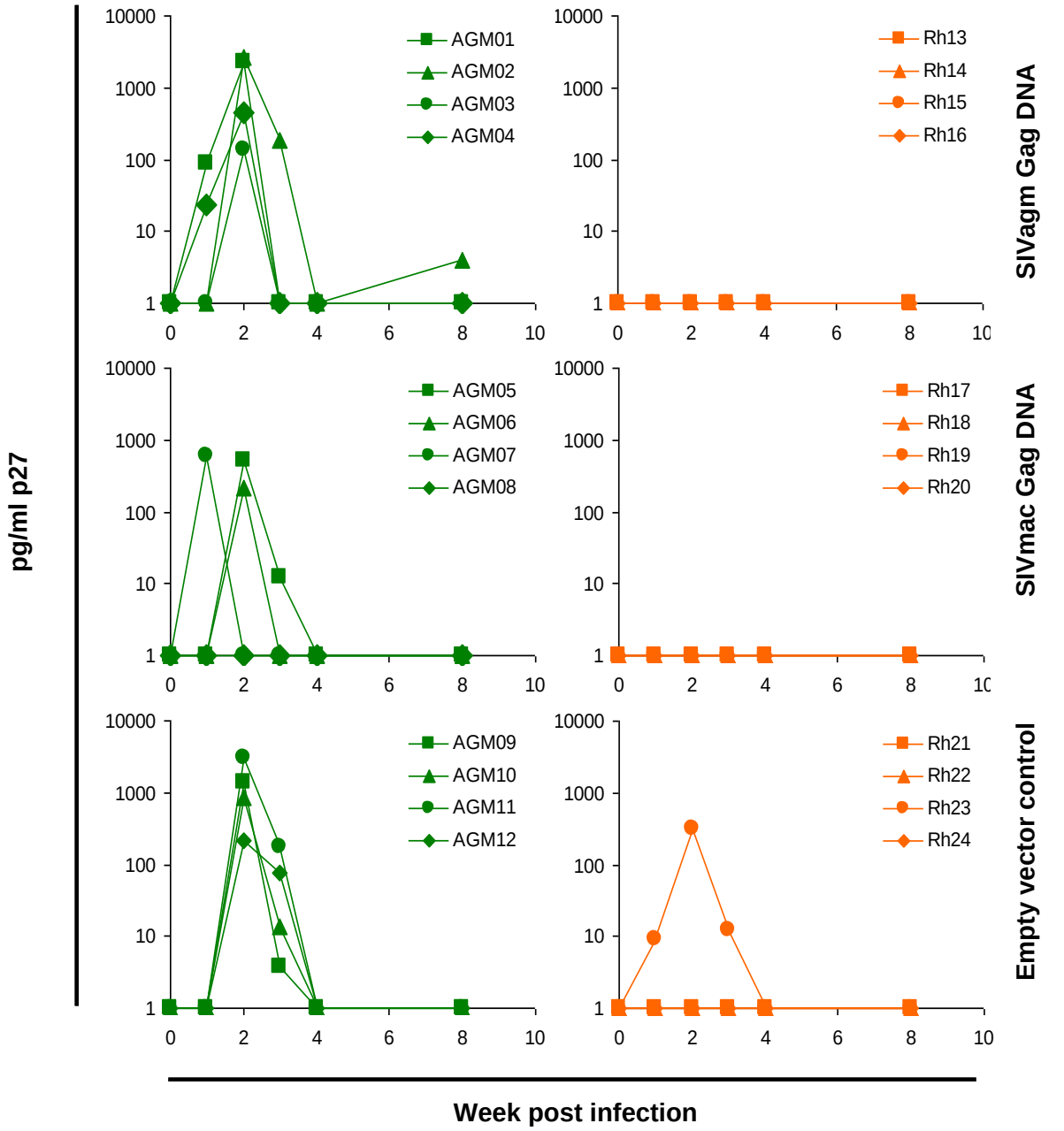

Figure 3.2: Plasma p27 protein levels in AGMs and Rhs involved in the Gag-DNA immunisation study at weeks 0, 1, 2, 3, 4 and 8 post infection with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The data are grouped according to immunogen (upper: SIVagmGag DNA, middle: SIVmacGag DNA, bottom: empty vector control).

3.3 Cytokine and chemokine levels during acute SIV infection

As the acute peak virus load, as measured by the number of genome copies or by levels of viral antigen, was even higher AGMs than in the rhesus, the lack of Gag-specific antibodies in AGMs could not be a result of low-level viremia in these particular animals. It was therefore decided to compare the induction of plasma cytokines and chemokines during the acute phase of exponential viral replication in the animals used in the SIV*gag*DNA immunisation study.

3.3.1 Differences between species

Levels of the cytokines and chemokines examined during acute SIV infection showed clear differences between AGMs and macaques, both between the two species and in some cases also between different immunisation groups within one species (figures 3.3.1a-p, tables 3.3.1a-c, 3.3.2a and 3.3.2b). Statistical analysis of the highest increases (peak value-baseline) were performed using the student's t-test ($p<0.05$), comparing each group with each of the remaining groups separately.

In rhesus macaques, there were remarkable increases between 66 and 240-fold in IFN alpha at week 1 of infection (figure 3.3.1a). This large increase was rapidly lost, but levels remained elevated compared to baseline at weeks 2 (data not shown) and 3 (table 3.3.2a). In AGMs, IFN-alpha levels only increased by a factor of two at week 2 p.i (data not shown) and were close to baseline at week 3p.i.(table 3.3.2a). The highest increases in IFN alpha levels of all rhesus macaques were significantly higher than those of all AGMs ($p=0.0001-0.025$).

The increase in Il-15 levels in the rhesus was also very pronounced (figure 3.3.1a), with a 20-30 fold median increase at week 1. Of note, baseline levels of IL-15 were higher in rhesus macaques compared with AGMs (table 3.3.1a). In the latter, increases of IL-15 were far less pronounced (2-4-fold, table 3.3.1b) and generally occurred later (average: 1.5 weeks p.i., table 3.3.1c). For IL-15, increases in all rhesus groups were significantly higher compared with all AGMs ($p= 0.000001-0.006$) and levels were significantly higher in the control rhesus (Rh21-24) compared with SIVmacGag-immunised rhesus (Rh17-20, $p= 0.012$).

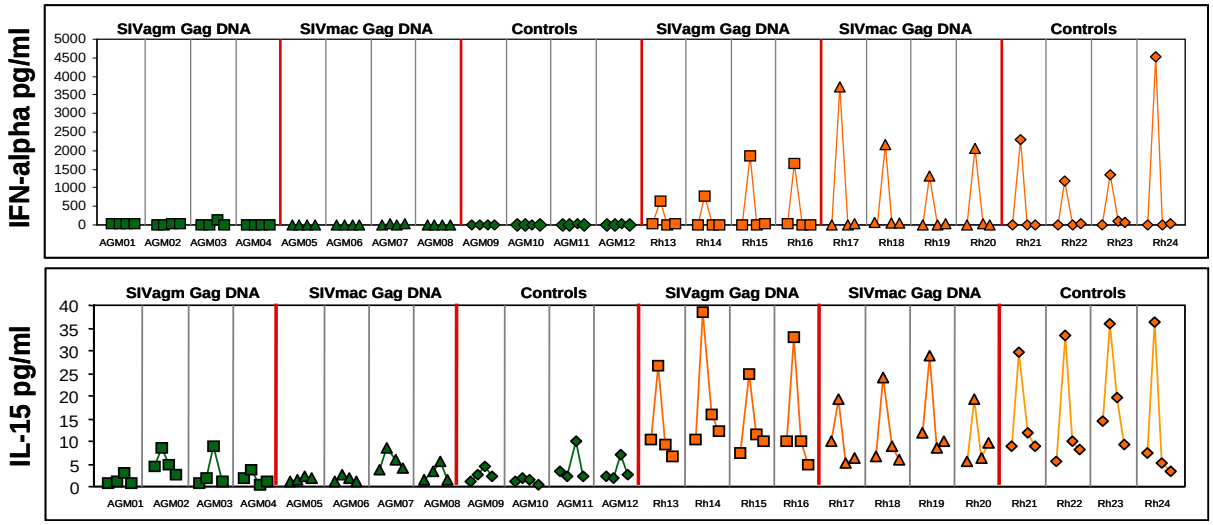

Figure 3.3.1a: Plasma IFN-alpha and IL-15 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

In comparison to IFN alpha levels, the increases in TNF alpha were far less pronounced (maximum: 25pg/ml in rhesus 17 at week 2 p.i., figure 3.3.1b). Baseline levels were lower in AGMs than in most of the macaques (table 3.3.1a) and increased approximately 2-fold in 7 out of 12 macaques (Rh 14, 16-20 and 23) at week 2 p.i. in most cases. Interestingly, only one rhesus (Rh23) of the control group had increased levels of TNF-alpha, whereas in the immunisation groups, 2 of 4 animals receiving SIVagmGag DNA and all receiving SIVmacGag DNA showed increases. In comparison, most AGMs showed no increased TNF-alpha levels, with the exception of AGMs 3 and 12, with detectable elevations at week 3 p.i and AGM 8, where the highest detectable level occurred at week 2 p.i.. In terms of groups, the only statistically significant difference was seen between the control macaques (Rh21-24) and the SIVmacGag-immunised macaques (Rh17-20, p=0.048), with higher levels in the latter.

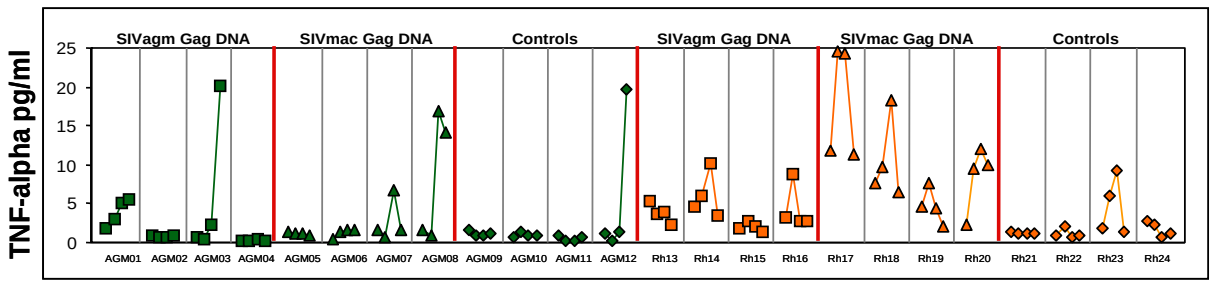

Figure 3.3.1b: Plasma TNF alpha levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

The levels of IL-18 in the macaques were similar (in terms of pattern, but not magnitude) to those of TNF-alpha (figure 3.3.1c). In three animals (Rh 15, 20, 22), there were marginal increases up to 20pg/ml plasma at week 2 p.i. Most other rhesus macaques peaked at high levels (50-100 pg/ml) at week 2 p.i.(Rh 13, 16, 17, -19). An exception was Rh 14, with fluctuating elevations peaking at both week 1 and week 3 p.i. Hardly any elevations of Il-18 could be detected in the AGMs (table 3.3.1b). Statistically significant differences between peak values occurred in both rhesus SIVagmGag and SIVmacGag immunisation groups (Rh13-16 and Rh 17-20, respectively) where levels were higher than in the rhesus control group (p=0.017 and 0.028, respectively). For these two groups, increases were also significantly more pronounced compared with the AGMs (p=0.01-0.02).

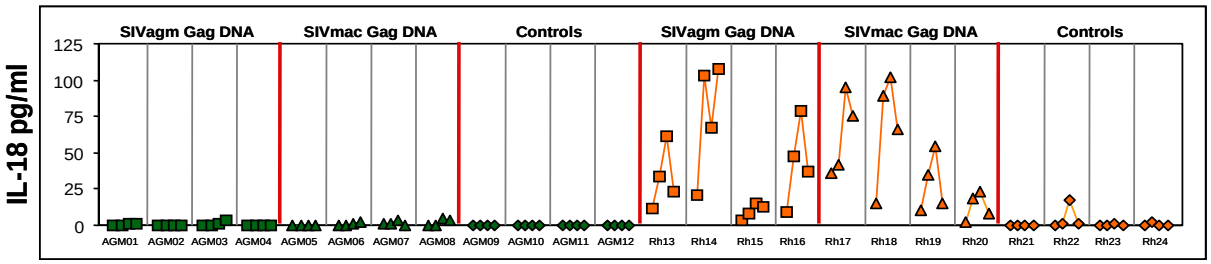

Figure 3.3.1c: Plasma IL-18 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

The increases in IFN-gamma levels (figure 3.3.1d) in the rhesus macaques at week 1 p.i. were moderate, except for rhesus 14 and 16 that had very high increases at this time. Levels in AGMs increased up to 14-fold (table 3.3.1b), but at an average of 0.5 weeks later (table 3.3.1c) than in rhesus and peak levels in AGMs (2.69-7.47pg/ml) were still lower than in rhesus (25.2-64.69pg/ml) due to lower baseline levels (table 3.3.1a). Statistically, interferon gamma peak values were higher in the SIVmacGag-immunised (Rh17-20) and control (Rh21-24) rhesus groups compared to all AGM groups (p=0.003-0.034), except for the SIVagmGag-immunised AGMs (AGM1-4), even though the average increase in rhesus macaques was moderate. The slightly higher peak levels in the SIVagmGag–immunised AGMs (AGM1-4) compared with the SIVmacGag–immunised AGMs (AGM5-8) was also significant (p=0.044; table 3.3.1b).

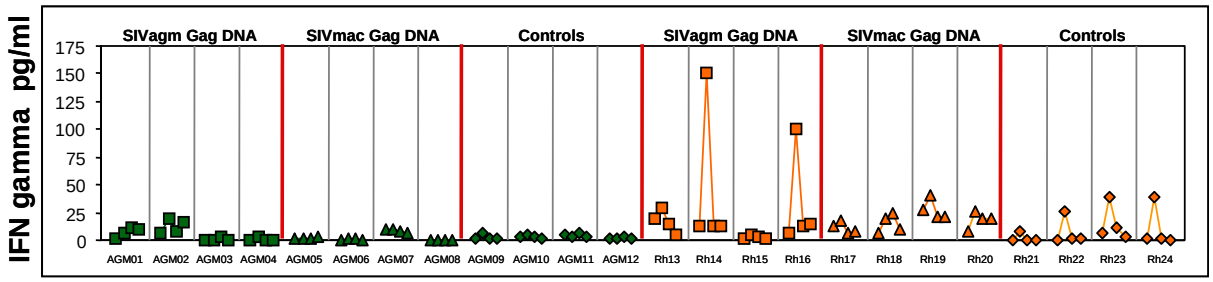

Figure 3.3.1d: Plasma IFN gamma levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Apart from rhesus 15, 17 and 21, IL-6 levels (figure 3.3.1e) in rhesus showed peak increases at week 1 p.i. Again, levels in AGMs remained at baseline (tables 3.3.1a and 3.3.1b). The increase in levels of IL-6 in SIVmacGag-immunised rhesus (Rh17-20) compared with all AGM immunisation groups was significant (p=0.014-0.028).

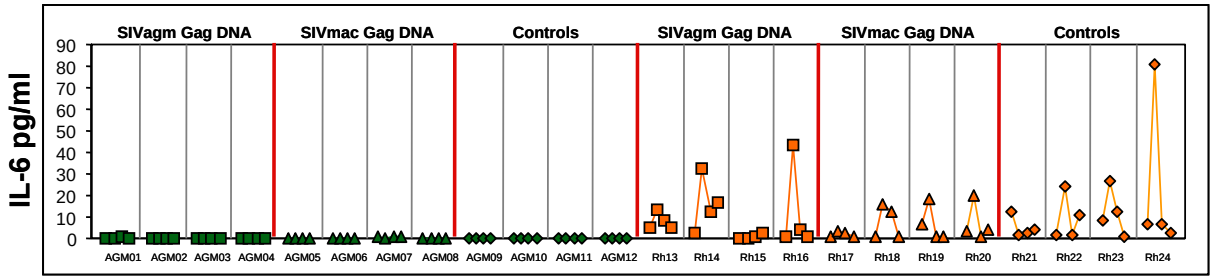

Figure 3.3.1e: Plasma IL-6 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Il-4 levels in the immunised rhesus groups fluctuated greatly (figure 3.3.1f). Rh 13 showed the highest detectable level at week 0, which were severely lower at weeks 1 and 3 p.i., but showed levels close to (though still lower than) baseline at week 2 p.i. In Rh 14, baseline levels were even higher than in Rh 13 and levels were elevated compared to baseline at week 1 p.i., but decreased thereafter to below-baseline levels. No change in IL-4 levels was observed in Rh 15. Rh 16 showed lower baseline levels compared to Rh 13 and 14 and showed a higher peak elevation than the latter two at week 1 p.i., which dropped back to slightly above baseline at week 2, but increased again at week 3 p.i. Rh 17 showed lower baseline levels than Rh 13, 14 and 16 and had only one detectable elevation at week 1 p.i. Rh 18 showed almost the same pattern as Rh17, except that the peak elevation was detectable at week 2 p.i. Rh 19 showed the highest baseline level of all rhesus macaques, which increased even more at week 1 p.i., but decreased thereafter to below-baseline levels at weeks 2 and 3 p.i. Rh 20 showed low baseline levels as Rh 15, 17 and 18, which increased at week 1 p.i., but stayed almost as elevated at week 2 p.i. before decreasing, though still being elevated above

baseline at week 3 p.i. Levels in the control group (Rh21-24) remained at baseline and once again, no increases in levels of this cytokine were seen in the AGMs. IL-4 peak levels differed significantly between SIVmacGag-immunised (Rh 17-20) and control rhesus (Rh 21-24; p=0.011), between the SIVmacGag-immunised rhesus (rh17-20) and all AGMs groups (p=0.009-0.038) and between the control rhesus (Rh21-24) and the SIVmacGag-immunised and control AGMs (AGM5-8 and AGM9-12, p=0.004 and P=0.025, respectively, table 3.3.1b).

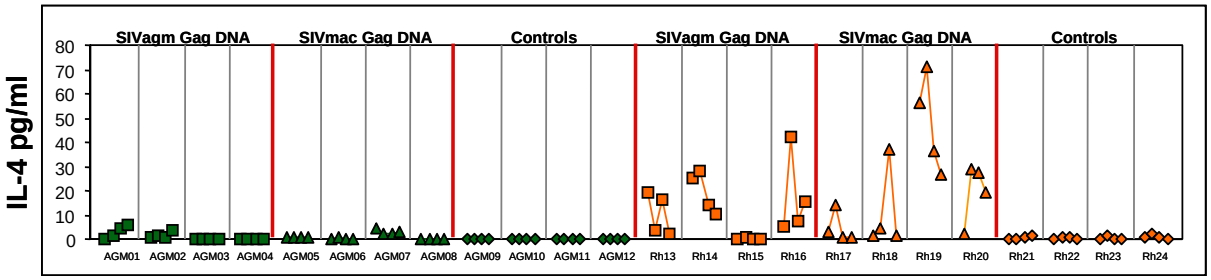

Figure 3.3.1f: Plasma IL-4 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

IL-5 levels (figure 3.3.1g) remained low in all macaques except for very small increases in rhesus 14 and 16 at week 1 p.i. and Rh 24 at week 3 p.i. AGMs 6 and 7 had very high baseline levels that dropped at week 1 p.i. but increased again at week 3 p.i. in AGM 7. Changes in IL-5 were only significant in the SIVmacGag-immunised rhesus (Rh17-20) and control rhesus (Rh 21-24) compared with AGM controls (AGM9-12, p=0.018 and 0.027, respectively), but these increases were only marginal and at the lower detection limit of the assay (table 3.3.1b).

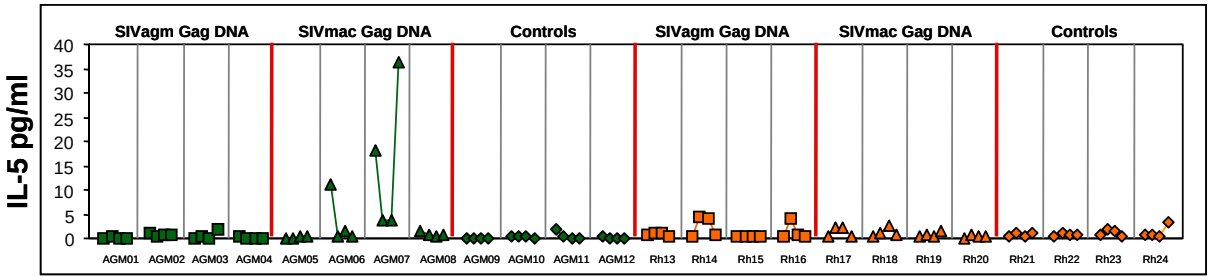

Figure 3.3.1g: Plasma IL-5 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

IL-12 levels showed vast fluctuations in both baseline and peak elevation levels in all AGM and rhesus groups, with no clearly identifiable patterns (figure 3.3.1h). Statistically, only the difference in peak levels between the SIVmacGag-immunised rhesus macaques (Rh17-20) and AGMs (AGM 5-8) was significant (p=0.006).

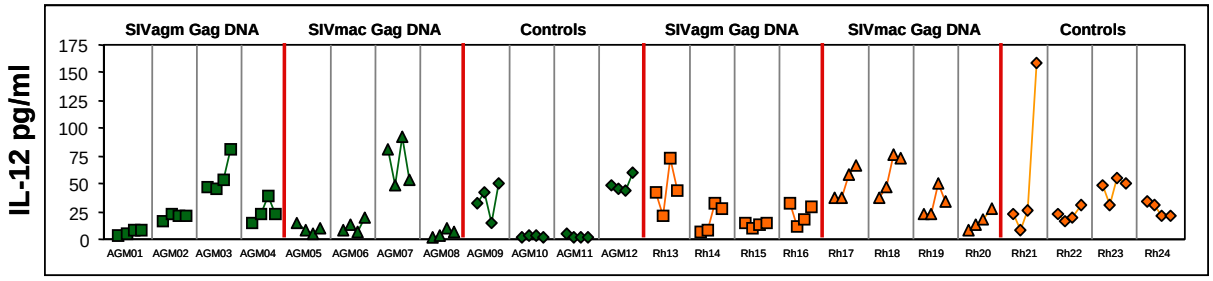

Figure 3.3.1h: Plasma IL-12 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Levels of IL-1Ra increased in all rhesus macaques at week 1 p.i. except for rhesus 15, whereas peak levels in AGMs were lower than in the rhesus macaques (table 3.3.1b, figure 3.3.1i) and occurred on average one week later (table 3.3.1c). Statistically, the peak levels were significantly higher in the control rhesus (Rh21-24) compared with all AGM groups (p=0.033-0.05) and in the SIVmacGag-immunised rhesus (Rh17-20) compared with the SIVmacGag-immunised and control AGMs (AGM5-8 and 9-12, p=0.038 and 0.039, respectively).

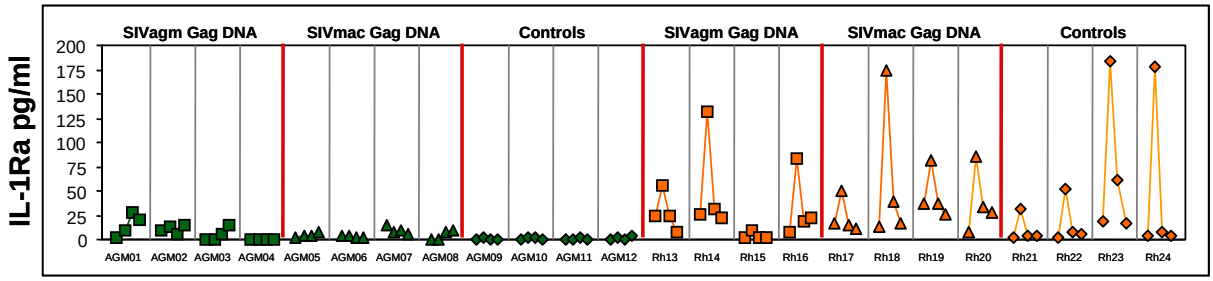

Figure 3.3.1i: Plasma IL-1Ra levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Baseline IL-2 levels and peak elevations remained constant in most AGMs but varied greatly in the rhesus macaques and (figure 3.3.1k), though the average levels for the group were within the same range (tables 3.3.1a and 3.3.1b). The exceptions in the AGMs were AGM 1, with elevated levels at week 2 p.i. and AGM 4 and 8 at week 3 p.i. that were clearly higher than baseline. The only statistically significant difference in peak levels was between the

control AGMs (AGM9-12) and the SIVagmGag-immunised AGMs (AGM1-4), with higher levels in the latter (p=0.035, table 3.3.1b).

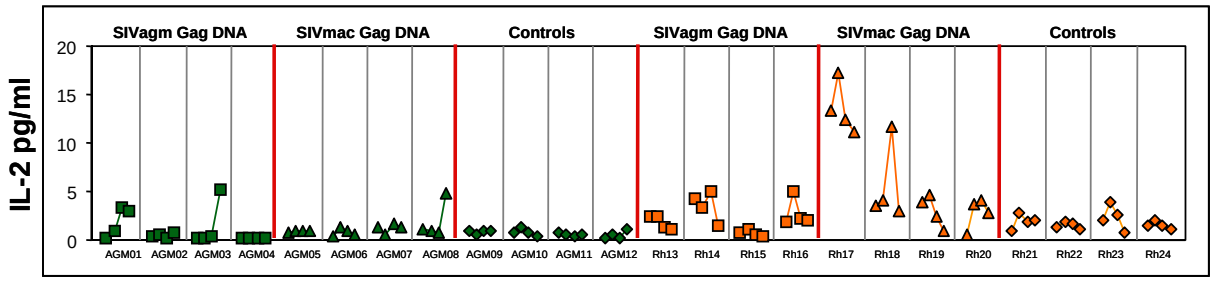

Figure 3.3.1k: Plasma IL-2 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Levels of sCD40L increased in all rhesus macaques except for rhesus 13, 15 and 24 (figure 3.3.1l). Levels peaked at week 1 p.i. in rhesus 16, 17 and 23, at week 2 p.i. in rhesus 14, 18 and 19 and were elevated at week 3 p.i. in rhesus 20 and. Again, baseline levels varied in both species with levels in the AGMs being on average higher than in the rhesus (146.75pg/ml vs. 108.94). Of note, baseline levels in both species were higher in the immunised than in the control groups (table 3.3.1a). In AGMs, increases in sCD40L occurred in all animals except for AGMs 4, 8 and 9. In AGMs 2, 4, 5 and 7, there was a decline in sCD40L levels at week 1 p.i., before levels increased again at week 2 p.i., with the highest levels being detected at week 3 p.i. in AGMs 5 and 7. Post-infection levels in AGM 4 remained below baseline. In AGMs 10-12, levels increased at week 1 p.i. but dropped again thereafter. In AGM 1, levels continued to increase, with the highest level detected at week 3. Although there was a tendency towards higher levels in AGMs than in rhesus, the only statistically significant difference in peak levels between the groups was seen between the SIVmacGag-immunised rhesus (Rh17-20) and the control AGMs (AGM9-12, p=0.018).

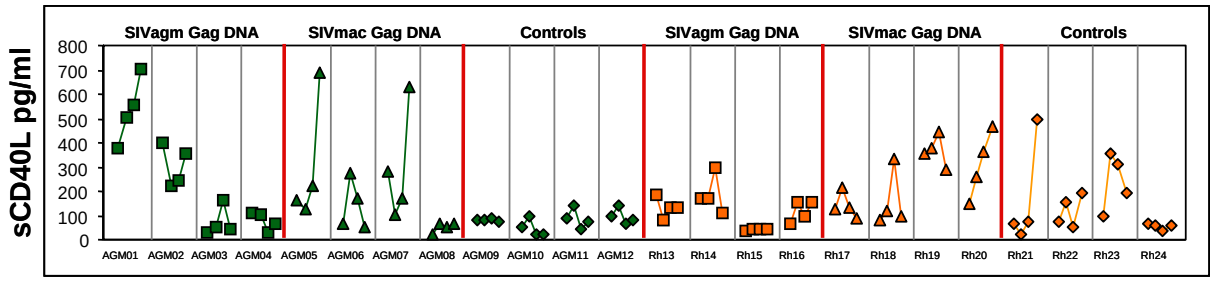

Figure 3.3.1l: Plasma sCD40L levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

MIP-1alpha levels (figure 3.3.1m) stayed close to baseline in Rh 14-16, 19, 20, 22 and 24. In the remaining rhesus macaques, baselines varied greatly. MIP-1 alpha levels peaked in Rh 13 and 17 at week 1 p.i., in Rh 23 at week 2 p.i. and showed the highest elevation in Rh 21 at week 3 p.i.; whereas in Rh 18, the level of MIP-1alpha was highest on the day of infection and dropped continuously thereafter. In AGMs, no changes were observed except for AGMs 8 and 9, which showed increases at week 3 p.i. There were no statistically significant differences between peak values of any groups.

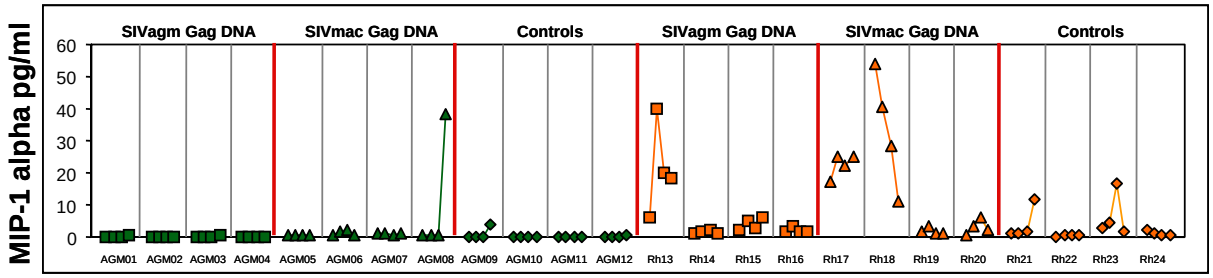

Figure 3.3.1m: Plasma MIP-1 alpha levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Baseline MIP-1beta levels (figure 3.3.1n) were also variable within the rhesus macaques. After infection, Rh 14, 16, 17, 20 and 22-24 showed peak plasma levels at week 1 p.i., Rh 18 at week 2 p.i. and Rh 21 showed the highest detectable levels at week 3 p.i., whereas in Rh15 levels stayed close to baseline and in Rh13, levels dropped below baseline at week 1 p.i. and then fluctuated at weeks 2 and 3. In both immunised rhesus groups, MIP-1beta levels tended to be higher than in the control rhesus. In AGMs, only marginal increases in MIP-1beta could be observed and only in the immunised groups, with levels in the control AGMs staying at baseline or declining slightly (table 3.3.1b). In AGM 2, levels peaked at 1 week p.i., in AGM 5, levels increased steadily with the highest recorded level at week 3 p.i., whereas in AGM 1, the peak elevation level was recorded at week 2 p.i. but declined thereafter. In AGM 7 levels dropped below baseline after infection. In all remaining AGMs (AGM3, 4, 6 and 8), MIP-1beta levels remained unchanged. The only statistically significant difference in peak elevation was seen between the SIVmacGag-immunised and control rhesus (Rh 17-20 and 21-24, respectively) compared with the control AGMs (AGM9-12, $p=0.042$ and 0.05, respectively), with higher levels in the former.

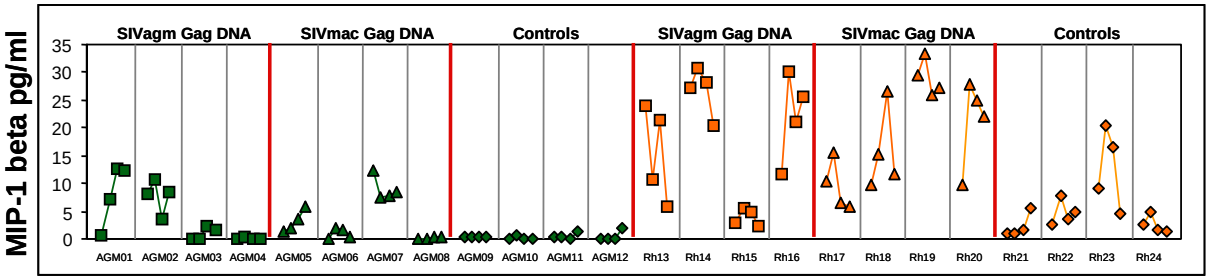

Figure 3.3.1n: Plasma MIP-1 beta levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

IFN-beta levels (figure 3.3.1o) also increased above baseline in most rhesus (Rh15, 17-21 and 24), with fluctuating dynamics. Levels also fluctuated in AGMs. In both species, baseline levels were higher in the immunised than in the control groups (table 3.3.1b). The overall response was higher in rhesus macaques compared to AGMs, with levels in the former increasing earlier than in the latter (week 1 p.i. vs. week 3 p.i., tables 3.3.1b and 3.3.1c). Statistically, the highest increases in IFN beta levels were significantly higher in the SIVmacGag-immunised rhesus (Rh17-20) compared to all AGM groups (p=0.0001-0.032) and compared to the control rhesus (Rh21-24, p=0.032). The levels of the latter were significantly higher than those of the SIVagmGag-immunised AGMs (AGM1-4, p=0.006). Of the AGMs, levels of the SIVmacGag-immunised (AGM5-8) and the controls (AGM9-12) were significantly higher than those of the SIVagmGag-immunised AGMs (AGM1-4, p=0.0001 and 0.009, respectively).

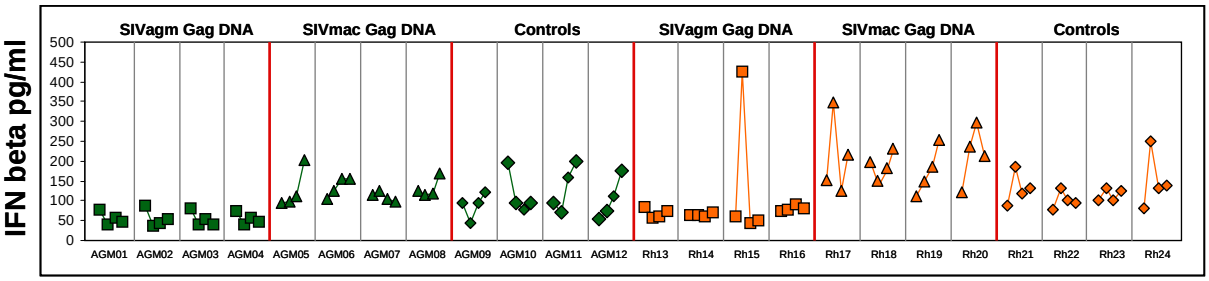

Figure 3.3.1o: Plasma IFN beta levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

No changes in the levels of G-CSF, GM-CSF and IL-1beta were observed in any of the animals with the exception of Rh 23, which showed an increase in G-CSF and IL-1beta and Rh 21, which showed increased levels of GM-CSF at week 3 p.i. Since IL-17 levels fluctuated only between 0-5pg/ml in all animals, these changes were not considered to be important.

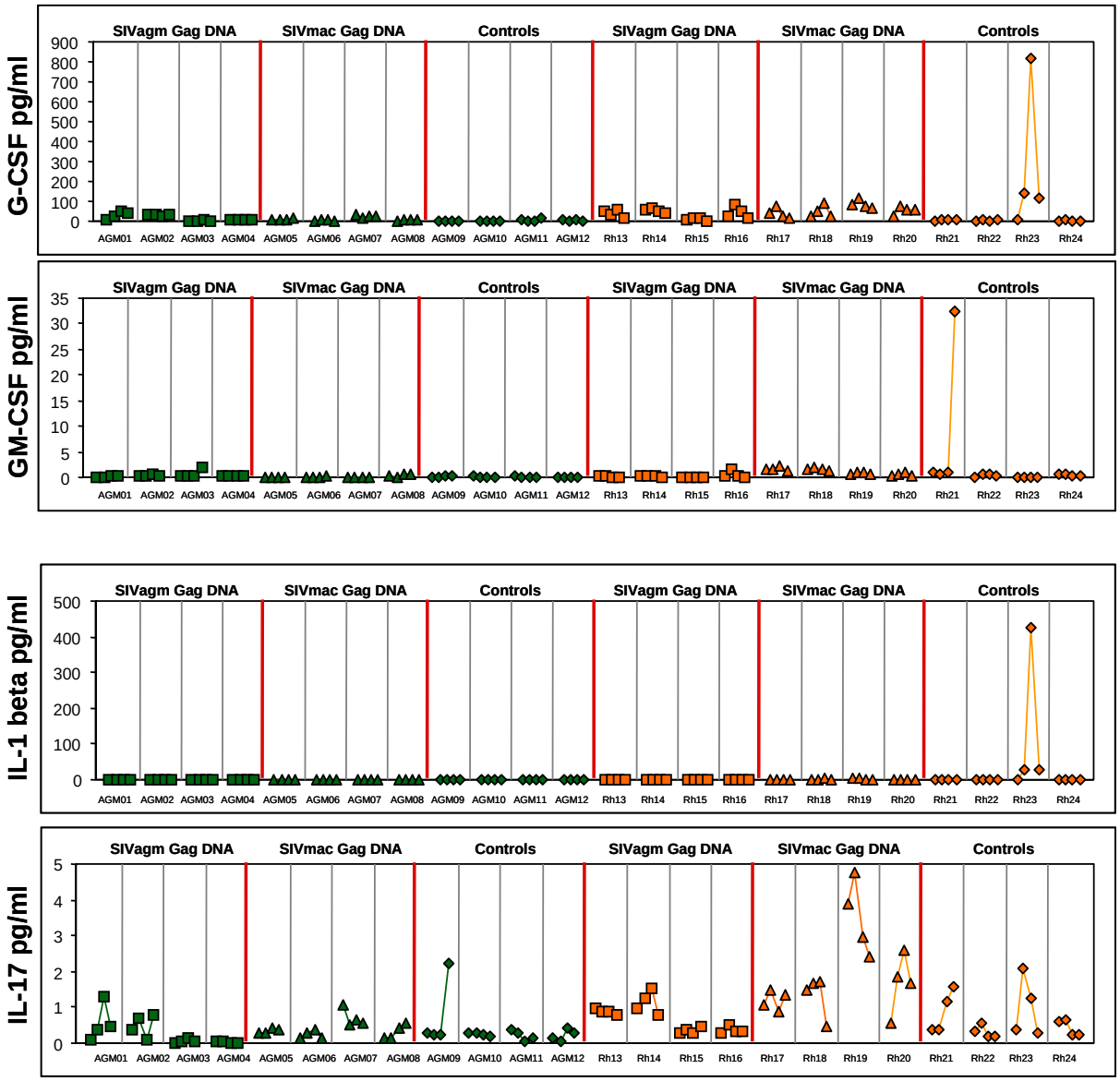

Figure 3.3.1p: Plasma G-CSF, GM-CSF, IL-1 beta and IL-17 levels in AGM and Rh from the immunisation study taken at 0, 1, 2, and 3 weeks after infection (from left to right) with SIVagm and SIVmac, respectively. Green: AGM samples. Orange: Rh samples. The animals are grouped according to immunogen.

Taken together, the patterns of changes in cytokine levels following SIV-infection in AGMs and rhesus macaques were complex. However, some clear patterns did emerge. Tables 3.3.1a-c summarise the median baseline (a), median highest recorded level (b) and the average time of maximum level (c) for each analyte within the different immunisation groups for each species.

Table 3.3.1a: Median baseline levels of plasma cytokines (before infection) in the different AGM and Rh immunisation groups and average baseline cytokine levels of all AGMs and Rhs.

Analyte	Detec-tion limit (pg/ml)	Median baseline (pg/ml)							
		AGM 1-4	AGM 5-8	AGM 9-12	Rh 13-16	Rh 17-20	Rh 21-24	Average AGM	Average Rh
G-CSF	0.46	5.90	4.29	4.38	38.31	33.77	2.10	4.86	24.72
GM-CSF	0.1	0.23	0.13	0.18	0.22	1.12	0.32	0.18	0.55
IFN-y	0.3	0.55	0.85	2.35	9.07	10.51	0.99	1.25	6.86
IL-1ß	0.16	0.14	0.47	0.09	0.87	0.79	0.24	0.24	0.63
IL-1ra	0.71	0.82	2.50	0.43	15.60	15.16	3.25	1.25	11.33
IL-2	0.73	0.20	0.99	0.67	2.08	3.65	1.38	0.62	2.37
IL-4	1.25	0.09	0.30	0.04	12.09	2.61	0.08	0.14	4.93
IL-5	0.26	0.19	6.41	0.37	0.37	0.32	0.60	2.32	0.43
IL-6	0.4	0.00	0.13	0.02	1.64	2.40	7.74	0.05	3.93
IL-12	1.11	15.88	10.84	18.65	23.41	29.76	28.49	15.12	27.22
IL-15	0.35	1.37	1.35	1.66	10.29	8.35	8.05	1.46	8.90
IL-17	0.13	0.07	0.22	0.29	0.61	1.27	0.37	0.19	0.75
MIP-1ß	5.84	0.36	0.64	0.23	17.76	10.01	2.64	0.41	10.13
MIP-1a	5.46	0.13	0.60	0.09	1.96	9.58	1.47	0.27	4.34
sCD40L	0.91	241.70	114.95	83.61	118.59	138.63	69.62	146.75	108.94
TNFa	0.86	0.77	1.54	1.15	3.92	6.15	1.54	1.15	3.87
IL-18	7.06	0.29	0.14	0.00	10.75	13.03	0.00	0.14	7.93
IFN-a	7	13.42	9.58	5.59	16.19	9.02	8.16	9.53	11.12
IFN-ß	2.3	79.35	108.93	93.98	70.43	137.03	85.77	94.09	97.74

Table 3.3.1b: Median peak elevation levels of plasma cytokines (post infection) in the different AGM and Rh immunisation groups and average peak cytokine levels of all AGMs and Rhs.

Analyte	Detec-tion limit (pg/ml)	Median maximum p.i. (pg/ml)							
		AGM 1-4	AGM 5-8	AGM 9-12	Rh 13-16	Rh 17-20	Rh 21-24	Average AGM	Average Rh
G-CSF	0.46	23.43	10.35	6.39	63.55	83.44	5.97	13.39	50.99
GM-CSF	0.1	0.51	0.15	0.13	0.27	1.46	0.55	0.27	0.76
IFN-y	0.3	7.47	2.69	5.66	64.69	25.20	32.01	5.28	40.63
IL-1ß	0.16	0.68	0.61	0.14	1.34	2.04	0.67	0.48	1.35
IL-1ra	0.71	14.75	7.82	2.07	68.95	83.55	115.58	8.21	89.36
IL-2	0.73	2.01	1.41	1.10	3.64	8.13	2.37	1.51	4.71
IL-4	1.25	2.02	0.76	0.04	22.05	32.96	1.41	0.94	18.81
IL-5	0.26	0.51	0.97	0.18	2.62	1.95	1.59	0.56	2.05
IL-6	0.4	0.05	0.19	0.01	22.84	16.92	25.32	0.09	21.69
IL-12	1.11	30.84	15.18	27.02	30.22	58.06	42.64	24.35	43.64
IL-15	0.35	6.07	3.96	5.86	29.68	21.79	34.74	5.30	28.74
IL-17	0.13	0.46	0.47	0.35	0.70	2.14	1.12	0.43	1.32
MIP-1ß	5.84	6.40	3.86	0.91	25.78	27.23	6.53	3.72	19.85
MIP-1a	5.46	0.37	1.72	0.28	4.68	15.61	6.47	0.79	8.92
sCD40L	0.91	261.01	453.91	118.52	146.06	389.36	275.35	277.82	270.26
TNFa	0.86	3.15	4.24	1.26	6.31	15.09	2.15	2.89	7.85
IL-18	7.06	0.52	2.87	0.00	69.69	74.51	1.27	1.13	48.49
IFN-a	7	44.77	15.39	18.79	1207.8	2100.4	1829.3	26.32	1712.5
IFN-ß	2.3	55.48	162.07	148.45	81.76	274.98	157.50	122.00	171.41

Table 3.3.1c: Average time point (week) of highest plasma cytokine (peak) level in the different AGM and Rh immunisation groups and average time point (week) of peak cytokine levels of all AGMs and Rhs.

	Average week of maximum p.i.							
Analyte	AGM 1-4	AGM 5-8	AGM 9-12	Rh 13-16	Rh 17-20	Rh 21-24	Average AGM	Average Rh
G-CSF	2.00	2.25	2.00	1.25	1.25	2.00	2.08	1.50
GM-CSF	2.25	2.50	1.75	1.25	1.75	1.50	2.17	1.50
IFN-y	1.50	1.50	1.50	1.00	1.25	1.00	1.50	1.08
IL-1ß	2.25	2.25	2.50	1.50	1.75	1.75	2.33	1.67
IL-1ra	2.25	2.25	2.00	1.00	1.00	1.00	2.17	1.00
IL-2	2.25	2.00	1.75	1.25	1.50	1.00	2.00	1.25
IL-4	2.25	2.50	2.00	1.25	1.25	1.75	2.25	1.42
IL-5	1.75	2.25	1.25	1.25	1.75	1.50	1.75	1.50
IL-6	2.00	2.75	1.75	1.50	1.00	1.50	2.17	1.33
IL-12	2.00	2.50	2.75	2.50	2.50	2.25	2.42	2.42
IL-15	1.50	1.50	1.75	1.00	1.00	1.00	1.58	1.00
IL-17	2.00	2.25	1.75	1.75	1.50	1.50	2.00	1.58
MIP-1ß	1.50	2.25	2.00	1.25	1.25	1.50	1.92	1.33
MIP-1a	2.00	1.75	2.00	1.75	1.25	1.75	1.92	1.58
sCD40L	2.25	2.50	1.25	2.00	2.00	2.50	2.00	2.17
TNFa	2.75	1.75	2.50	1.50	1.50	1.75	2.33	1.58
IL-18	2.50	2.00	2.00	2.25	2.00	2.00	2.17	2.08
IFN-a	1.75	2.25	2.25	1.00	1.00	1.00	2.08	1.00
IFN-ß	2.25	2.50	3.00	2.25	2.25	1.00	2.58	1.83

In summary, in most cases, peak levels of cytokines were detected at week 1 or 2 post infection in rhesus macaques, corresponding to or shortly before the peak of plasma viremia, whereas in AGMs, the highest levels of cytokines were seen mainly at weeks 2 and 3. In addition, most post-infection cytokine peak levels were higher in rhesus macaques than in AGMs.

3.3.2 Post-peak plasma cytokine elevations

In many cases, post-peak cytokine levels did not return to baseline but instead remained elevated at week 3 p.i. in the rhesus macaques and AGMs, though in the latter, the week 3 p.i. values often corresponded to the highest detectable levels. Tables 3.3.2a and 3.3.2b summarise the median cytokine elevation above baseline (a) and the median fold change of cytokine baseline levels at week 3 p.i. (b) in each group of animals.

On average, levels of plasma IL-12, sCD40L, IL-18, IFN alpha and IFN beta were still highly elevated (+10-36pg/ml) and plasma IL-1 beta, IL-5 and MIP-1 alpha showed moderate elevations (0.13-0.69pg/ml) compared to baseline in the rhesus macaques. Interestingly, levels of the remaining markers decreased to levels below initial baseline levels (table 3.3.2a). Although most of these reductions were only slight (0.1-1.67pg/ml), the average level of G-CSF in the macaques was of 5.97pg/ml lower at week 3 p.i. than on the day of infection. For most of the cytokines showing post-peak elevations, the levels were only approximately twice those at baseline. The one exception was IFN alpha, where the levels was on average 27-fold higher than baseline, although this is due to the fact that baseline levels in the control rhesus (Rh21-24) were close to zero. Consequently, the elevation above baseline of only 0.02pg/ml gave a fold-increase of more than 77.

In comparison, although average plasma cytokine levels in the AGMs at week 3 p.i. were mainly elevated above baseline, most changes were only moderate (0.06-2pg/ml). The exceptions were IL-1 Ra (+4.14pg/ml), IL-12 (+4.28pg/ml), sCD40L (+57.75pg/ml) and IFN beta (+26.93pg/ml). In contrast, IL-6 levels returned to baseline (±0pg/ml) and levels of IL-5 and IL-15 were slightly decreased (-0.2 and -0.28 pg/ml, respectively).

Table 3.3.2a: Median elevation levels of plasma cytokines at 3 weeks post infection in the different AGM and Rh immunisation groups and average cytokine elevation levels in all AGMs and Rhs.

	Median elevation (pg/ml) above baseline at week 3 p.i.							
Analyte	**AGM 1-4**	**AGM 5-8**	**AGM 9-12**	**Rh 13-16**	**Rh 17-20**	**Rh 21-24**	**Average AGM**	**Average Rh**
G-CSF	0.41	1.83	-1.58	-11.64	-9.33	3.07	0.22	-5.97
GM-CSF	0.22	0.05	-0.08	-0.10	-0.18	0.00	0.06	-0.10
IFN-y	4.51	0.17	-0.22	-0.01	-0.97	-0.67	1.49	-0.55
IL-1ß	0.19	0.05	0.02	0.03	-0.01	0.36	0.09	0.13
IL-1ra	10.26	2.23	-0.06	-1.50	-1.55	0.74	4.14	-0.77
IL-2	1.62	0.18	-0.10	-0.78	-1.28	-0.27	0.57	-0.78
IL-4	1.38	0.02	-0.03	-7.49	-0.81	-0.05	0.46	-2.78
IL-5	-0.12	-0.47	-0.25	-0.04	0.36	0.58	-0.28	0.30
IL-6	0.02	0.01	-0.02	1.27	-0.13	-6.16	0.00	-1.67
IL-12	6.65	0.53	5.67	0.46	24.34	5.71	4.28	10.17
IL-15	-0.55	0.13	-0.17	-1.00	-1.16	-1.97	-0.20	-1.38
IL-17	0.21	0.03	0.01	-0.07	-0.36	-0.13	0.08	-0.19
MIP-1ß	0.84	0.27	0.48	-3.61	-0.11	0.45	0.53	-1.09
MIP-1a	0.25	-0.05	0.21	1.97	0.51	-0.39	0.14	0.69
sCD40L	-11.41	198.58	-13.92	-20.81	-13.52	107.27	57.75	24.31
TNFa	1.88	0.51	-0.07	-0.82	-0.79	-0.28	0.77	-0.63
IL-18	0.32	0.99	0.00	19.36	22.40	0.02	0.43	13.93
IFN-a	-0.39	4.14	2.35	1.66	12.56	20.96	2.03	11.73
IFN-ß	-32.67	47.46	65.99	-2.85	78.37	32.95	26.93	36.16

Table 3.3.2b: Median fold change of plasma cytokine levels at 3 weeks post infection in the different AGM and Rh immunisation groups and average fold-change in cytokine levels in all AGMs and Rhs.

	Median fold change of cytokine level at week 3 p.i.							
Analyte	AGM 1-4	AGM 5-8	AGM 9-12	Rh 13-16	Rh 17-20	Rh 21-24	Average AGM	Average Rh
G-CSF	0.20	0.94	-0.50	-0.42	-0.13	2.72	0.21	0.72
GM-CSF	2.44	0.72	-0.67	-0.50	-0.16	0.12	0.83	-0.18
IFN-y	3.92	0.50	-0.05	-0.09	0.09	2.85	1.45	0.95
IL-1ß	1.51	0.13	0.16	0.05	0.01	2.37	0.60	0.81
IL-1ra	9.50	1.39	-0.09	0.07	-0.05	0.57	3.60	0.20
IL-2	7.97	0.32	-0.15	-0.49	-0.14	-0.18	2.71	-0.27
IL-4	2.62	0.55	-0.45	-0.48	-0.06	-0.62	0.91	-0.38
IL-5	0.61	0.07	-0.65	-0.12	1.74	2.19	0.01	1.27
IL-6	8.17	0.06	-0.79	3.07	-0.11	-0.68	2.48	0.76
IL-12	0.64	0.68	0.16	0.01	0.87	0.22	0.49	0.37
IL-15	-0.32	0.04	-0.08	-0.10	-0.12	-0.17	-0.12	-0.13
IL-17	1.39	0.09	0.19	-0.01	-0.06	-0.38	0.56	-0.15
MIP-1ß	8.03	3.11	1.46	-0.21	0.06	0.16	4.20	0.00
MIP-1a	1.42	-0.10	4.47	0.94	0.05	0.06	1.93	0.35
sCD40L	0.31	1.66	-0.16	-0.02	-0.01	1.34	0.60	0.44
TNFa	1.16	0.97	0.02	-0.22	-0.09	-0.17	0.72	-0.16
IL-18	1.75	6.09	1.89	2.64	2.02	77.41	3.24	27.36
IFN-a	0.00	0.47	0.44	0.09	1.72	3.88	0.31	1.90
IFN-ß	-0.39	0.42	0.70	-0.03	0.59	0.36	0.24	0.30

3.4 Development of biomarker realtime PCRs

3.4.1 Sequencing of AGM and rhesus biomarker cDNAs

A large number of AGM biomarker cDNAs were sequenced and are described in the appendix. Each sequence was aligned to the corresponding rhesus cDNA and human genomic DNA sequence and the resulting "gaps" within the AGM and rhesus cDNA sequences were defined as predicted exons. Using this information, primers for realtime PCRs were designed to bind within predicted exons flanking intron sequences of sufficient length to likely exclude the amplification of genomic DNA.

3.4.2 Establishing and optimising assays with AGM and rhesus cDNAs

The efficiencies of the biomarker realtime PCRs using the newly-designed primers were established by running serial dilutions of cDNA from stimulated AGM and rhesus PBMCs in one assay. Each diluted sample was run in triplicate using a Stratagene MX3005P realtime

PCR cycler. The means and standard deviations were calculated from the ct values of each triplicate sample, using the same threshold for all the samples in the same experiment on the same plate. Using Excel, mean ct values were then plotted against the dilutions and the correlation coefficient R^2 (i.e. the efficiency of the PCR) was calculated (figure 3.4.2a).

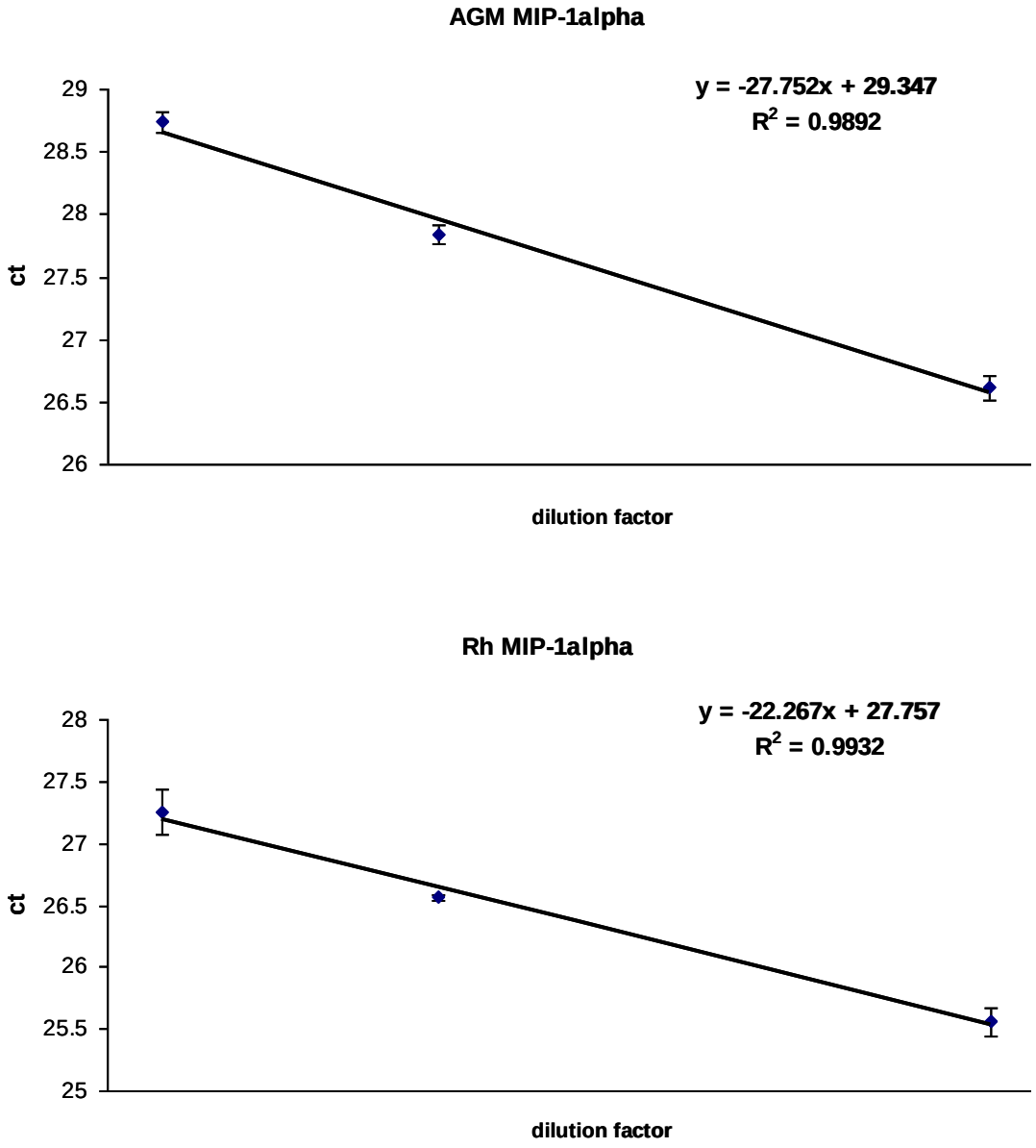

Figure 3.4.2a: Determination of efficiencies of the MIP-1 alpha realtime PCRs using the newly-designed primers. Serial dilutions of cDNA from stimulated AGM and rhesus PBMCs were run in one assay. Each diluted sample was run in triplicate using a Stratagene MX3005P realtime PCR cycler. The means and standard deviations were calculated from the ct values of each triplicate sample, using the same threshold for all the samples in the same experiment on the same plate. Using Excel, mean ct values were then plotted against the dilutions and the correlation coefficient R^2 (i.e. the efficiency of the PCR) was calculated. Hence, the efficiency of the realtime PCR for MIP-1 alpha cDNA was 98.92% for AGM and 99.32% for rhesus macaques, within ct ranges of 25-29. The efficiency of the MIP-1 alpha realtime PCR for both species was therefore 99.12% ± 0.2.

For example, the efficiency of the realtime PCR for MIP-1 alpha cDNA was 98.92% for AGM and 99.32% for rhesus macaques, within ct ranges of 25-29 (figure 3.4.2a). The efficiency of the MIP-1 alpha realtime PCR for both species was therefore calculated to be 99.12% ± 0.2.

Although the efficiency of each realtime PCR would ideally be 100%, to facilitate running the assays using the same master mix (apart from the primers) and under the same cycling conditions, efficiencies higher than 95% were considered acceptable providing the deviation between the two species was less than 1%. In fact, the interspecies deviation between the AGM and rhesus sample measurements was 0.02-0.5%. Efficiencies of the individual biomarker realtime PCRs are shown in table 3.4.2a.

Table 3.4.2: Calculated efficiencies for realtime PCRs with cDNA of stimulated AGM and rhesus PBMCs. Efficiencies are the mean of AGM and rhesus. The standard deviations for the efficiencies were between 0.02-0.5%.

Biomarker	Realtime efficiency %	Biomarker	Realtime efficiency %	Biomarker	Realtime efficiency %
FoxP3	98.50	IL-15	99.89	IP-10	98.26
GAPDH	98.99	IL-16	98.20	IRF-7	99.88
GM-CSF	99.68	IL-18	99.87	MCP-1	100.00
IFN alpha	99.06	IL-1Ra	99.93	MIP-1 alpha	99.12
IFN beta	98.09	IL-2	97.43	MIP-1 beta	99.67
IFN gamma	99.66	IL-22	99.46	PD-1	100.00
IL-1 alpha	98.01	IL-4	99.56	PDL-1	98.89
IL-10	97.94	IL-5	99.96	PDL-2	97.74
IL-12 alpha	99.99	IL-6	96.34	RANTES	98.37
IL-12 beta	98.59	IL-7	95.02	TNF alpha	99.69
IL-13	99.33	IL-8	96.38	VEGF	98.63

The method initially used for synthesising cDNA from cell pellet RNA yielded only very low amounts of cDNA. As a result, only 0.1µl of cDNA synthesised from 2.2-fold diluted RNA (due to the reaction setup) could be used in the 25µl realtime PCR reaction, a 555-fold dilution of the original RNA. This was necessary because higher amounts of the cDNA synthesis reaction (containing EDTA) completely inhibited the assay.

Although this high dilution of cDNA was not a problem for the determination of biomarkers are expressed in cell types present at high frequencies in the PBMCs of AGMs and rhesus macaques, the method proved to be too insensitive for the determination of rarer biomarkers. In addition, the RNA in PBMC pellets of $5x10^5$ cells taken from AGMs and macaques used in the SIV*gag*DNA immunisation study stored for 3.5 years was found to be partially degraded.

The poor quality and small quantity of the stored samples taken during the acute phase of infection as part of the SIV*gag*DNA immunisation study made it likely that the realtime PCR assay results would be unreliable. It was therefore decided to prepare RNA from fresh PBMC samples taken from chronically infected AGMs and macaques and compare the different biomarker levels at later time points of infection.

PBMCs were therefore isolated from selected animals in the primate colony and immediately lysed for subsequent RNA isolation. This ensured large quantities of high quality RNA. Furthermore, to facilitate measurement of rarer biomarkers, a commercial one-step SYBR Green RT-PCR kit (Quantitect, Qiagen) was used that allows specific cDNA synthesis for each designated biomarker prior to the realtime PCR in the same reaction. In this way, efficiency of the cDNA synthesis was increased and dilution of the RNA was avoided.

The reactions for each biomarker were considered contamination-free if H_2O controls yielded 'no ct' or the dissociation curve of the H_2O controls that did give positive ct values were unequivocally distinct from those of sample wells (figure 3.4.2b). The copy number/GAPDH ratio was calculated for each biomarker measurement.

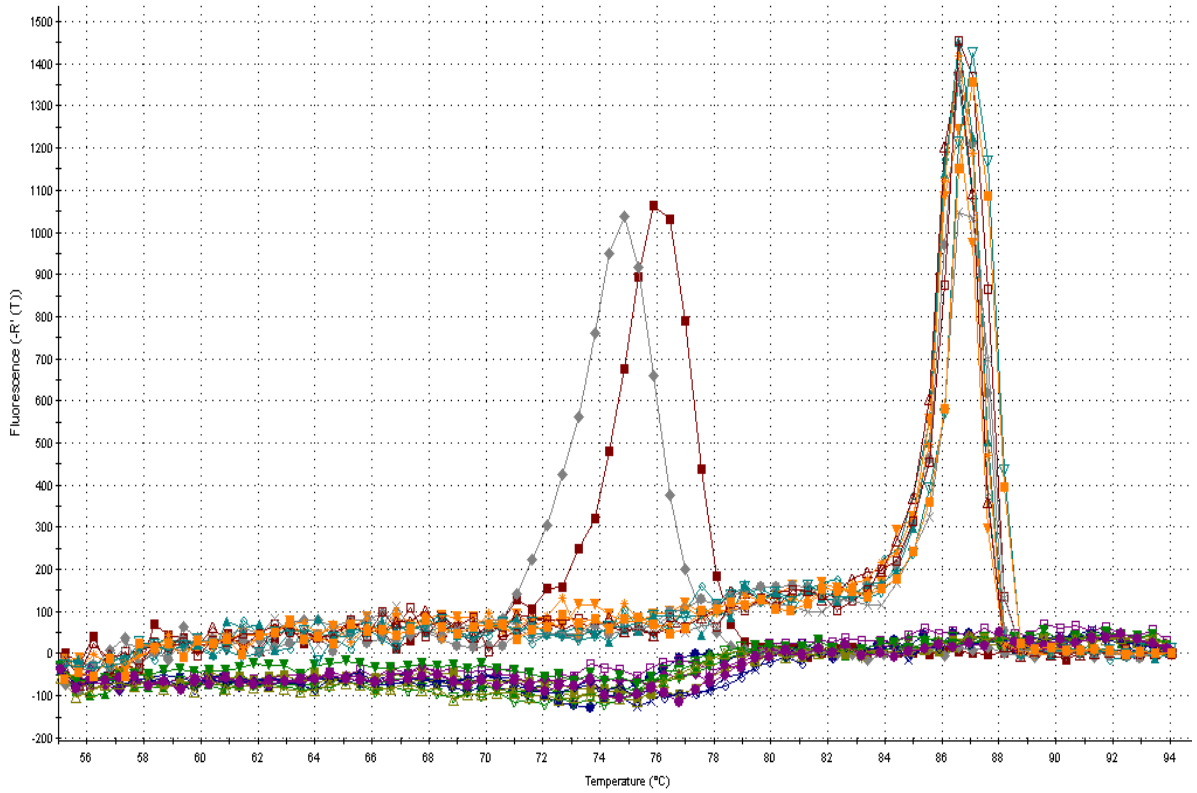

Figure 3.4.2b: Dissociation curves of six samples and two H_2O controls (grey and dark red) that did give positive ct values in the realtime PCR are shown. The dissociation curves of the latter are unequivocally distinct from those of the sample wells (dissociation at 74-75°C (primer dimers) versus 87°C (PCR products)). Hence, the H_2O controls were considered negative.

3.5 Biomarker expression levels in PBMCs from chronically SIV-infected AGMs and rhesus macaques

The expression levels of the sequenced biomarkers relative to GAPDH were determined at the RNA level in PBMCs from chronically SIV-infected AGMs and rhesus macaques. Levels of IFN beta, IL-1 beta, IL-12 alpha, IL-12 beta and IL-5 could not be detected or gave ct values well outside the efficiency range. For the remaining markers, RNA expression levels in 2-6 animals of each species for each individual marker were determined. For all biomarkers whose expression level had been determined in four or more animals of each species, statistical analyses (two-tailed t-test; $p \leq 0.05$) were performed.

Biomarkers of the type 1 interferon response (figure 3.5a):

IFN alpha levels of in AGM (n=6) and rhesus (n= 4) did not significantly differ, whereas the expression level of IRF-7 was significantly higher (p=0.014) in rhesus (n=4) compared to the AGMs (n=6).

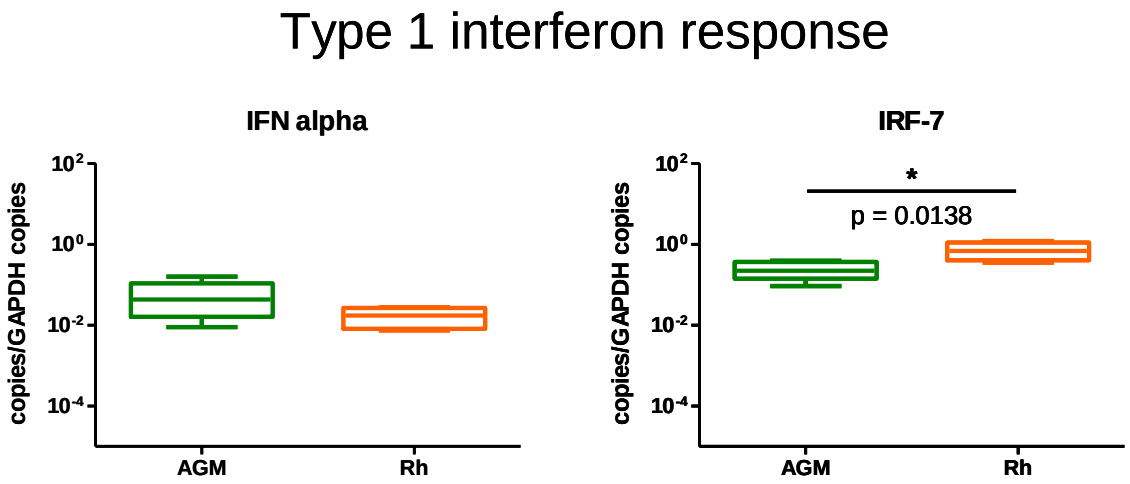

Figure 3.5a: Biomarker/GAPDH ratios of IFN alpha, and IRF-7 in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

Th1-type cytokines (figure 3.5b):

There was no significant difference between AGMs (n=6) and rhesus (n=4) for levels of IFN gamma expression, although for TNF alpha, there was a slight tendency for higher levels in rhesus (n=2) compared with AGMs (n=3). Expression levels of TNF alpha were generally higher than those of IFN gamma.

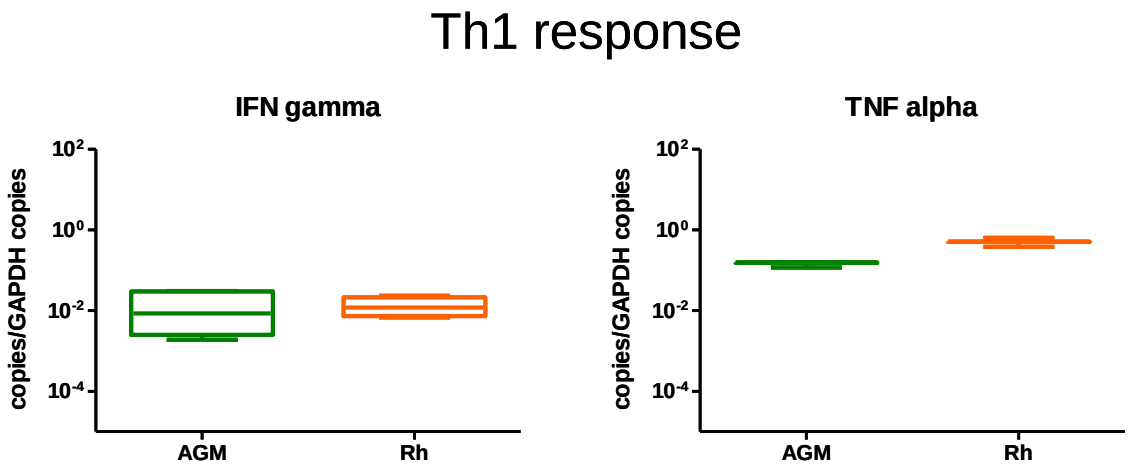

Figure 3.5b: Biomarker/GAPDH ratios of IFN gamma and TNF alpha in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

Th2-type cytokines (figure 3.5c):

Levels of IL-4 expression did not significantly differ between the two species (n=5), as was the case for IL-13 (AGM: n=3; Rh: n=2). However, levels of IL-6 expression were significantly higher (p=0.0422) in AGMs (n=5) compared with rhesus (n=5), whereas levels of IL-10 expression were significantly higher (p=0.0017) in the rhesus (n=4) compared to the AGMs (n=6).

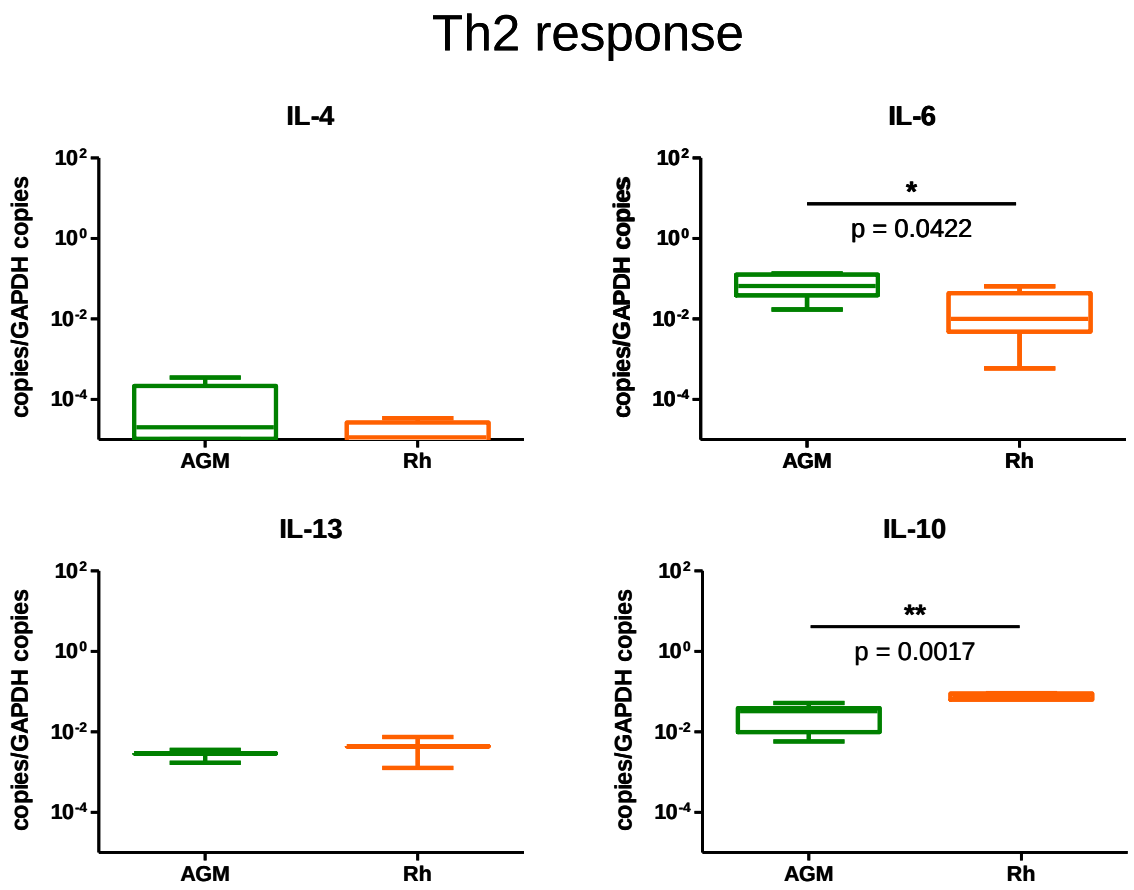

Figure 3.5c: Biomarker/GAPDH ratios of IL-4, IL-6, IL-10 and IL-13 in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

General markers for a proinflammatory response (figure 3.5d):

Expression levels of GM-CSF, IL-22, IL-1 alpha and IL-18 were similar in AGMs (n=3) and rhesus (n=2), although there was a tendency for higher IL-15 levels in rhesus (n=2) compared with AGMs (n=3). For levels of IL-7, IL-16 and VEGF, the difference was not significant (n=5). Levels of both IL-16 and VEGF expression were generally higher in both species compared with the other markers.

General markers for anti-inflammatory responses (figure 3.5e, page 68):

The difference of FoxP3 expression in AGMs (n=5) and rhesus (n=5) was not significant and levels of IL-1Ra were also similar (AGM: n=3; Rh: n=2).

Biomarkers for T-cell exhaustion (figure 3.5f, page 68):

Levels of PD1 and PD-L2 expression did not differ between the two species (n=5), whereas levels of PD-L1 were significantly higher (p=0.0051) in AGMs (n=5) compared to rhesus (n=5)

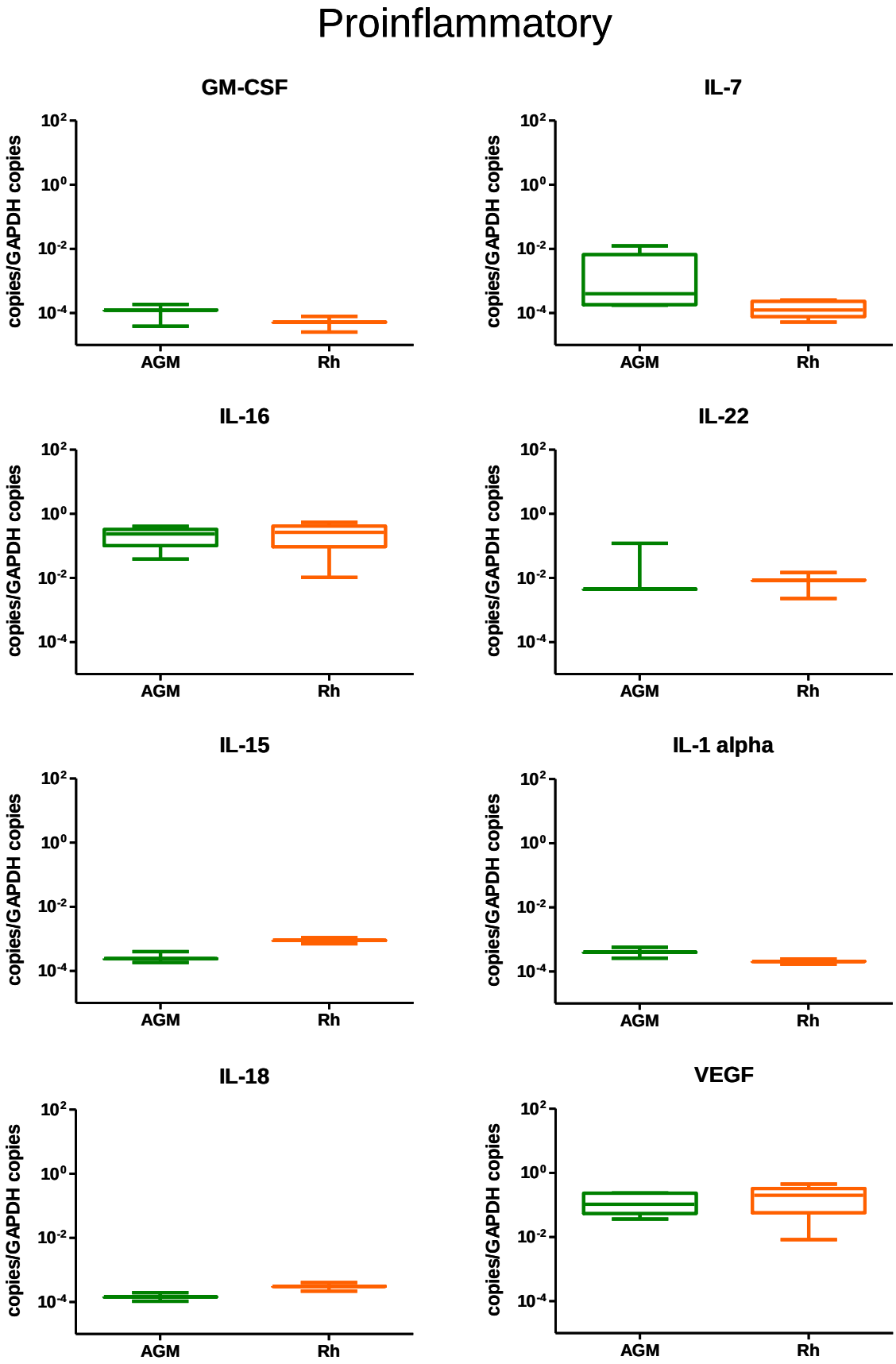

Figure 3.5d: Biomarker/GAPDH ratios of GM-CSF, IL-7, IL-16, IL-22, IL-15, IL-1 alpha, IL-18 and VEGF in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

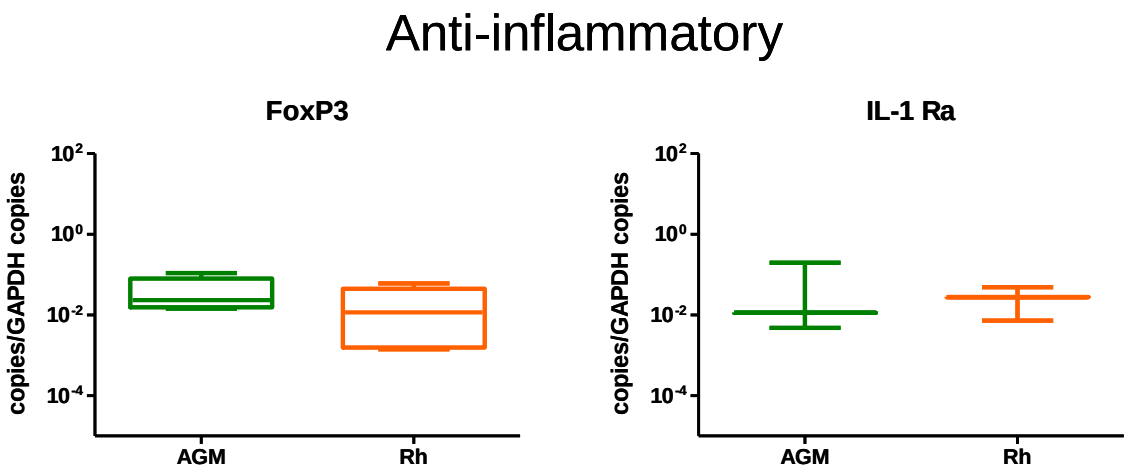

Figure 3.5e: Biomarker/GAPDH ratios of FoxP3 and IL-1Ra in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

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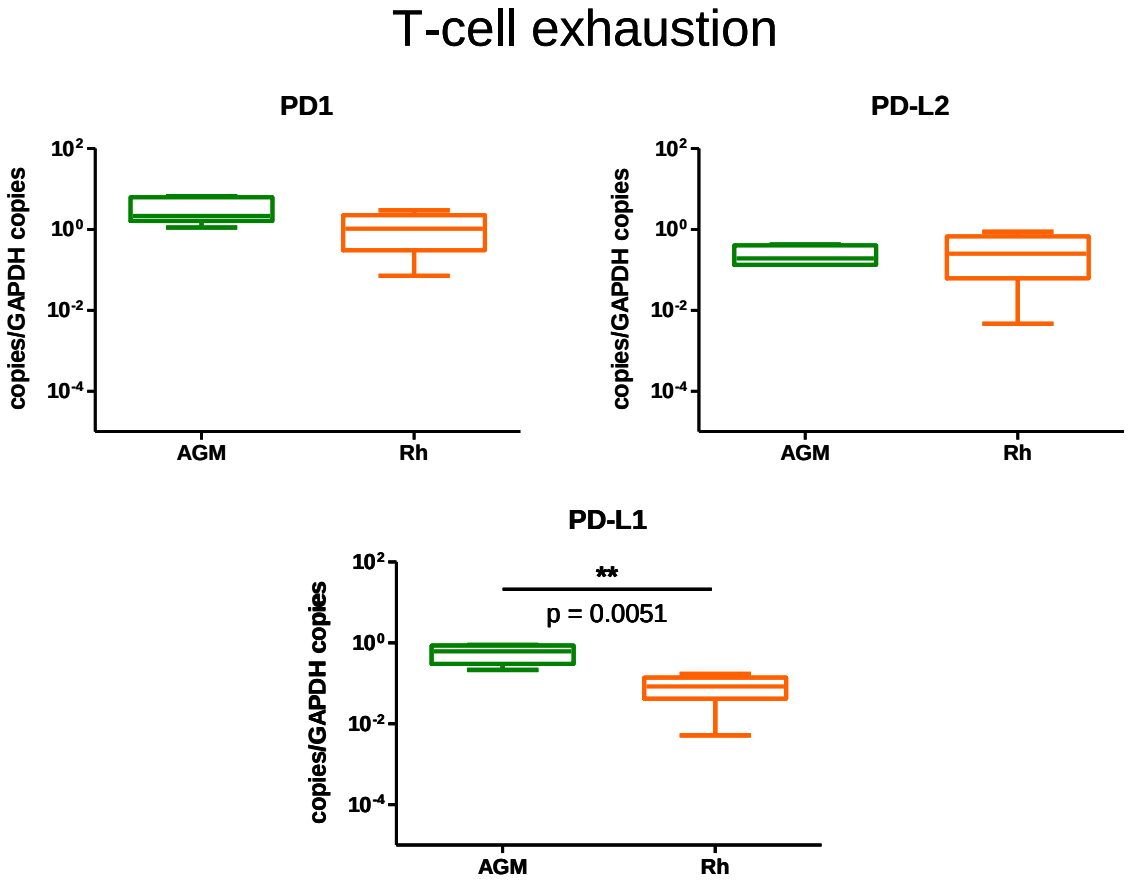

Figure 3.5f: Biomarker/GAPDH ratios of PD1, PD-L1 and PD-L2 in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

Chemokines (figure 3.5g):

Levels of IL-8 expression were similar in AGMs, whereas the levels of IP-10 expression appeared to be at least two orders of magnitude higher in the rhesus macaques (n=2) compared to the AGMs (n=3). This however has yet to be confirmed by increasing sample numbers. MIP-1 alpha levels also tended to be higher in rhesus. MCP-1, MIP-1 beta and RANTES expression did not differ between AGMs (n=5) and rhesus (n=5).

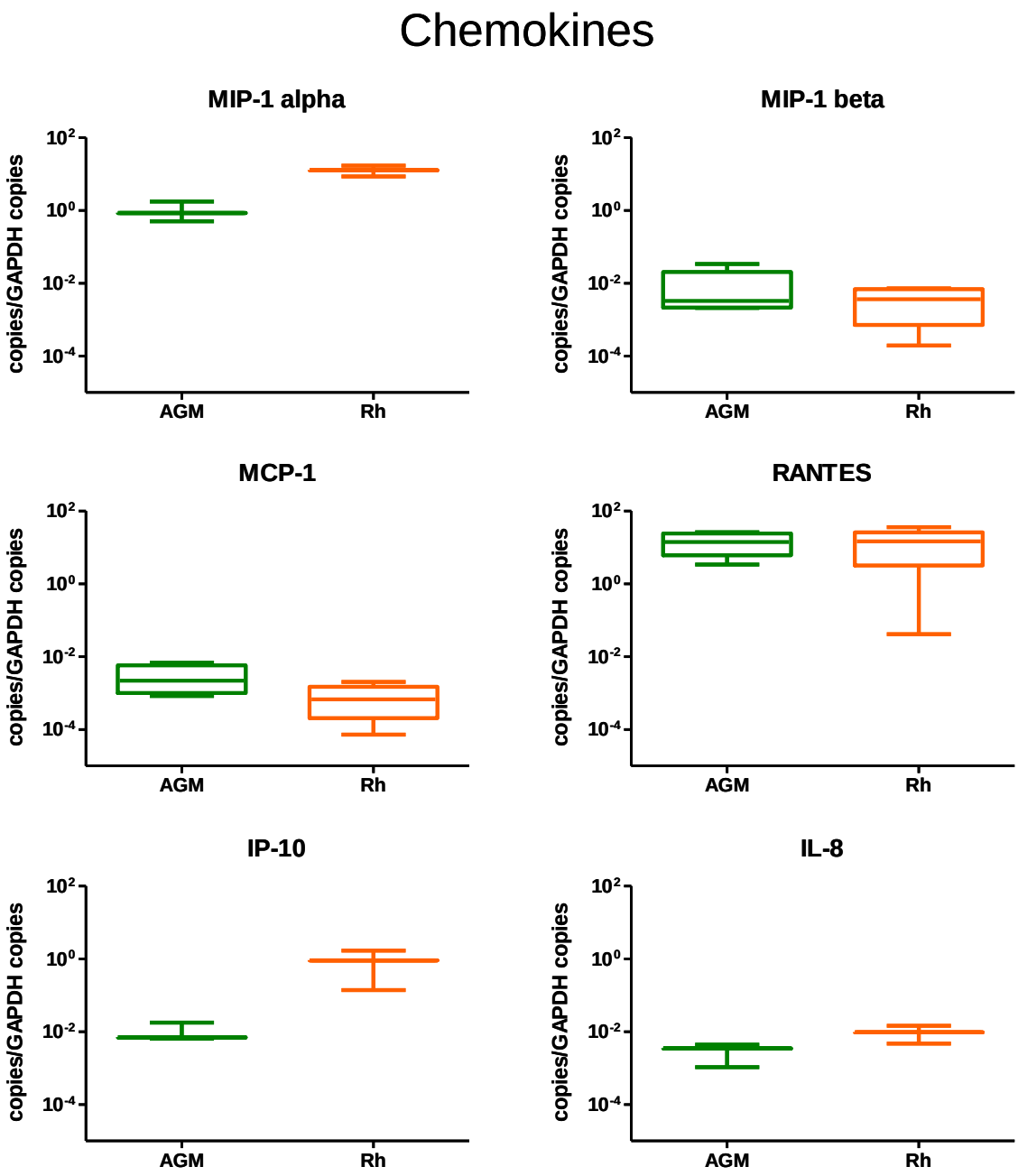

Figure 3.5g: Biomarker/GAPDH ratios of MIP-1 alpha, MIP-1 beta, MCP-1, RANTES, IP-10 and IL-8 in PBMCs of chronically SIV-infected AGMs and rhesus determined with realtime RT-PCR.

3.6 Interferon regulatory factor 7 sequence

Differences in the sequence of the IRF-7 protein in the sooty mangabey natural hosts of SIVsm and the disease-susceptible heterologous macaque had previously been implicated as a possible reason for the lack of ability of sooty mangabey pDCs to produce high levels of IFN alpha upon activation of intracellular TLRs [198]. As the AGM IRF-7 sequence had, for the first time, been sequenced to allow development of the realtime RT-PCR, this was compared to that of other primates. Figure 3.6 shows the aligned IRF-7 amino acid sequences of four different species (human, rhesus, sooty mangabey and African green monkey). It is striking that the valine at position 268 within the transactivation domain is shared by AGMs and sooty mangabeys but not by humans and macaques.

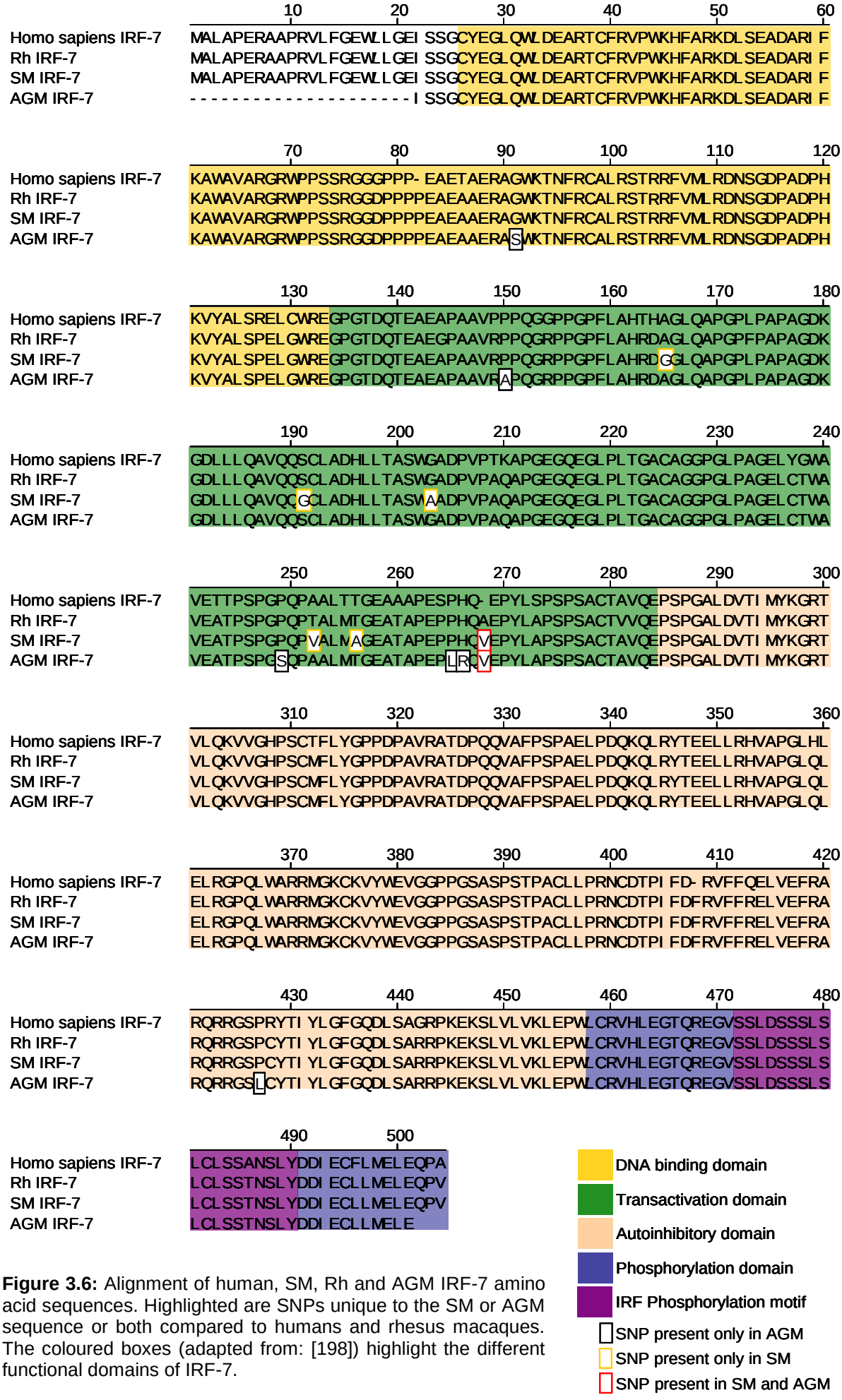

Figure 3.6: Alignment of human, SM, Rh and AGM IRF-7 amino acid sequences. Highlighted are SNPs unique to the SM or AGM sequence or both compared to humans and rhesus macaques. The coloured boxes (adapted from: [198]) highlight the different functional domains of IRF-7.

3.7 Interferon alpha ELISPOT

PDCs are particularly important for eliciting host responses to viral infections and secrete IFN alpha via a pathway that requires the activation of IRF-7 [199, 200]. In the case of HIV/SIV infection, the activation of IRF-7 is facilitated by TLR7 located in the endosome. In order to compare the responsiveness of AGM pDCs to infection with that of rhesus, PBMCs from SIV-naïve AGMs and rhesus were stimulated with the TLR7 agonist R-848 or with AT2–inactivated SIVmac239, SIVagm and HIV-1 in IFN alpha Elispot plates. SUPT1 cell–derived microvesicles were included as a negative control. In addition, an inhibitor of TLR7 (IRS661) and chloroquine, which prevents the acidification of the endosome, were added to demonstrate the dependency of TLR7 stimulation for IFN alpha secretion in pDCs. As the number of spots obtained with cells incubated with R-848 was in agreement with the frequency of pDCs in AGMs and rhesus in a preliminary experiment (data not shown), spot counts with the different stimulants were normalised to the number obtained with R-848.

The number of reactive cells in PBMCs stimulated with AT-2 inactivated SIVagm, SIVmac or HIV were similar for rhesus and AGMs although the variation within each species was rather high (figure 3.7a). Addition of IRS661 and chloroquine to cultures stimulated with AT-2 inactivated SIVagm/SIVmac abrogated IFN alpha production. The involvement of TLR7 signaling and endosomal acidification in SIV-induced IFN alpha secretion was therefore confirmed. Furthermore, the lack of IFN alpha secretion in cultures exposed to microvesicles confirmed HIV/SIV as the trigger for pDC avtivation. These data therefore demonstrate that the same frequency of pDCs is activated in AGMs and rhesus PBMC upon exposure to HIV/SIV.

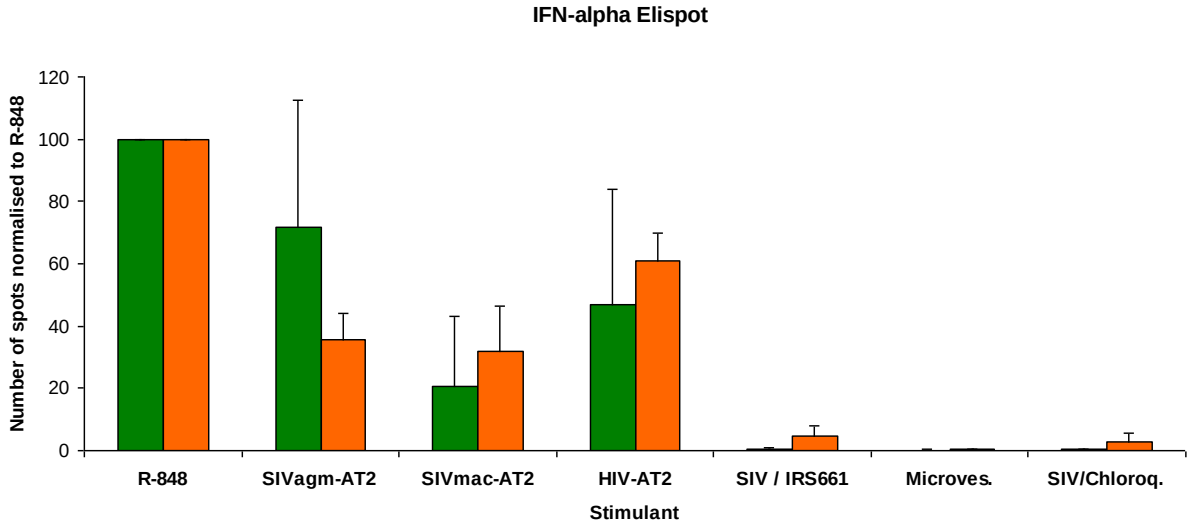

Figure 3.7a: Numbers of IFN alpha secreting cells (spots) in PBMCs of SIV-naïve AGMs (n=4) and rhesus (n=3) stimulated with R-848, AT2-inactivated SIVmac239, SIVagm or HIV-1, or in the presence of endosomal TLR signalling inhibitors (IRS661, chloroquine). Negative control: SUPT1 cell–derived microvesicles. Spot numbers are shown normalised to those detected with R-848 stimulation.

Spot sizes are a measure of the quantity of IFN alpha secretion at the level of individual pDC and for AGMs and rhesus these were found to lie within a similar range (27 to 100 µm^2, figure 3.7b). Overall, there was no difference between spot sizes for the two species after stimulation with R-848 or with AT2-inactivated SIVagm, SIVmac or HIV.

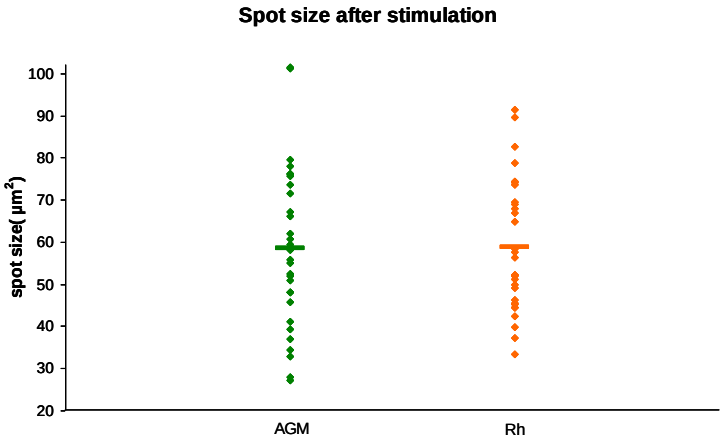

Figure 3.7b: Size of spots formed by IFN alpha secreting cells in PBMCs of SIV-naïve AGMs (n=4) and rhesus (n=3) stimulated in IFN-alpha Elispot plates with R-848, AT2–inactivated SIVmac239, SIVagm or HIV-1.

However, when comparing the cytokine activity (a measurement derived from the size and the intensity of each individual pixel) of the spots between the two species, a clear difference between AGM and rhesus pDCs stimulated with HIV or SIV was seen (figure 3.7c). This effect was not observed when cells were stimulated with R-848 (not shown).

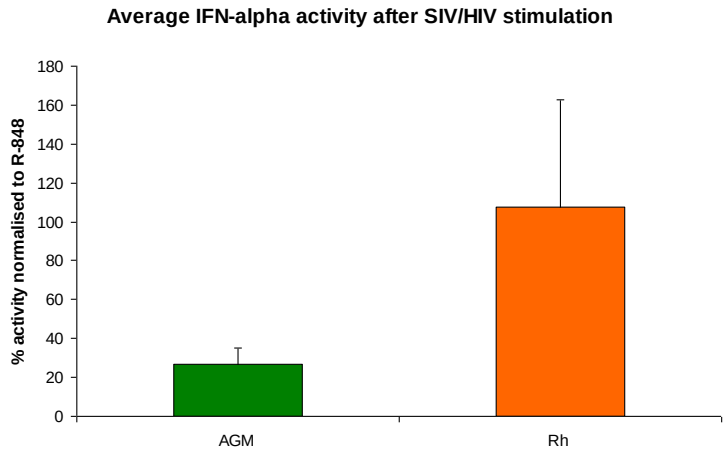

Figure 3.7c: Cytokine activity of IFN alpha secreting pDCs of SIV-naïve AGMs (n=4) and rhesus (n=3) stimulated on IFN-alpha Elispot plates with AT2–inactivated SIVmac239, SIVagm and HIV-1. Cytokine activities are shown normalised to those detected with R-848 stimulation in each individual animal.

3.8 Quantification of antibodies and B-cells

3.8.1 Total IgG ELISA

Since the endosomal TLR-signalling in AGMs appeared to be impaired upon HIV/SIV stimulation similarly to sooty mangabeys, this was suggested to be a general mechanism and not a property of pDCs only. One other cell that can be activated by TLR signals is memory B-cells. Since the absence of anti-Gag antibodies in SIV-infected AGMs had been suggested to be due to a general mechanism rather than one specific for SIV, it was hypothesised that the polyclonal activation of memory B-cells via TLRs could also be impaired in AGMs after SIV-infection. This would also be reflected in the total amount of IgG present in plasma. Thus, the total IgG in the plasma of AGMs and rhesus before and during acute and chronic infection with SIV were compared by ELISA.

In an initial experiment, plasma samples from each monkey before and during the chronic stage of SIV-infection (6 months p.i.) were titrated and allowed to dry on plates before incubation with the IgG-specific, enzyme-labelled second antibody. This allowed and optimum dilution beyond the prozone (presumably caused by steric hindrance at high protein concentrations) to be determined (figure 3.8.1a).

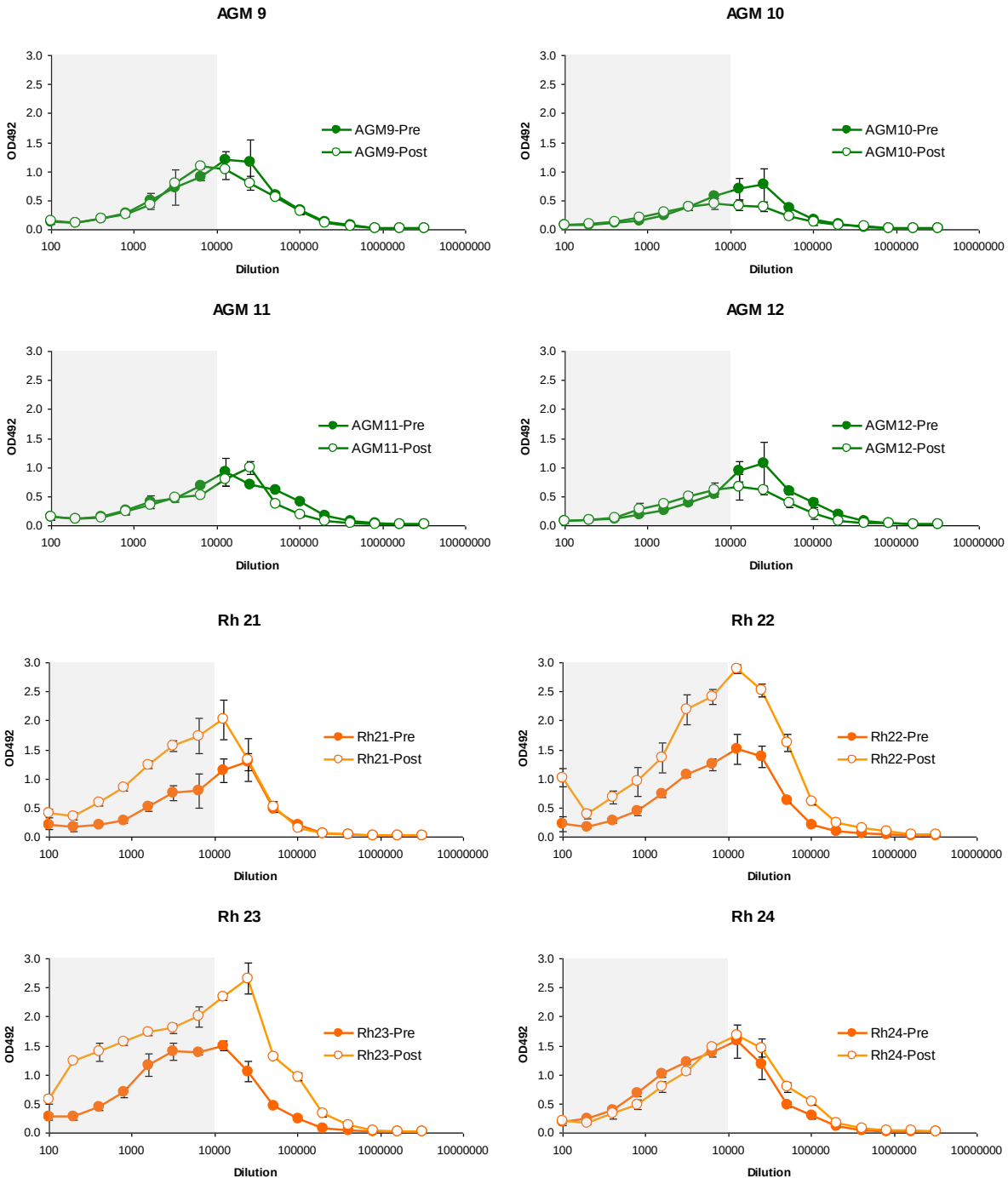

Figure 3.8.1a: Titration of plasma samples taken from individual Rhs and AGMs before and after infection with SIVmac and SIVagm, respectively. Titrated plasma was left to dry overnight and IgG detected using an anti-human IgG antibody. Filled symbols: pre-infection samples. Empty symbols: post-infection samples. Grey: Dilutions at which a prozone effect was evident.

Plasma samples from all four AGMs gave lower signals than those of the four macaques. In AGMs 9, 10 and 12, peak values were higher with the plasma samples taken before infection, in contrast to the macaques in which peak values were higher after infection. As the peaks for all plasma samples was seen at a dilution of around 1:10.000, this was chosen for the analysis

of sequential plasma samples from these animals. Figure 3.8.1b shows the data obtained using plasma over the course of infection diluted 1:10.000.

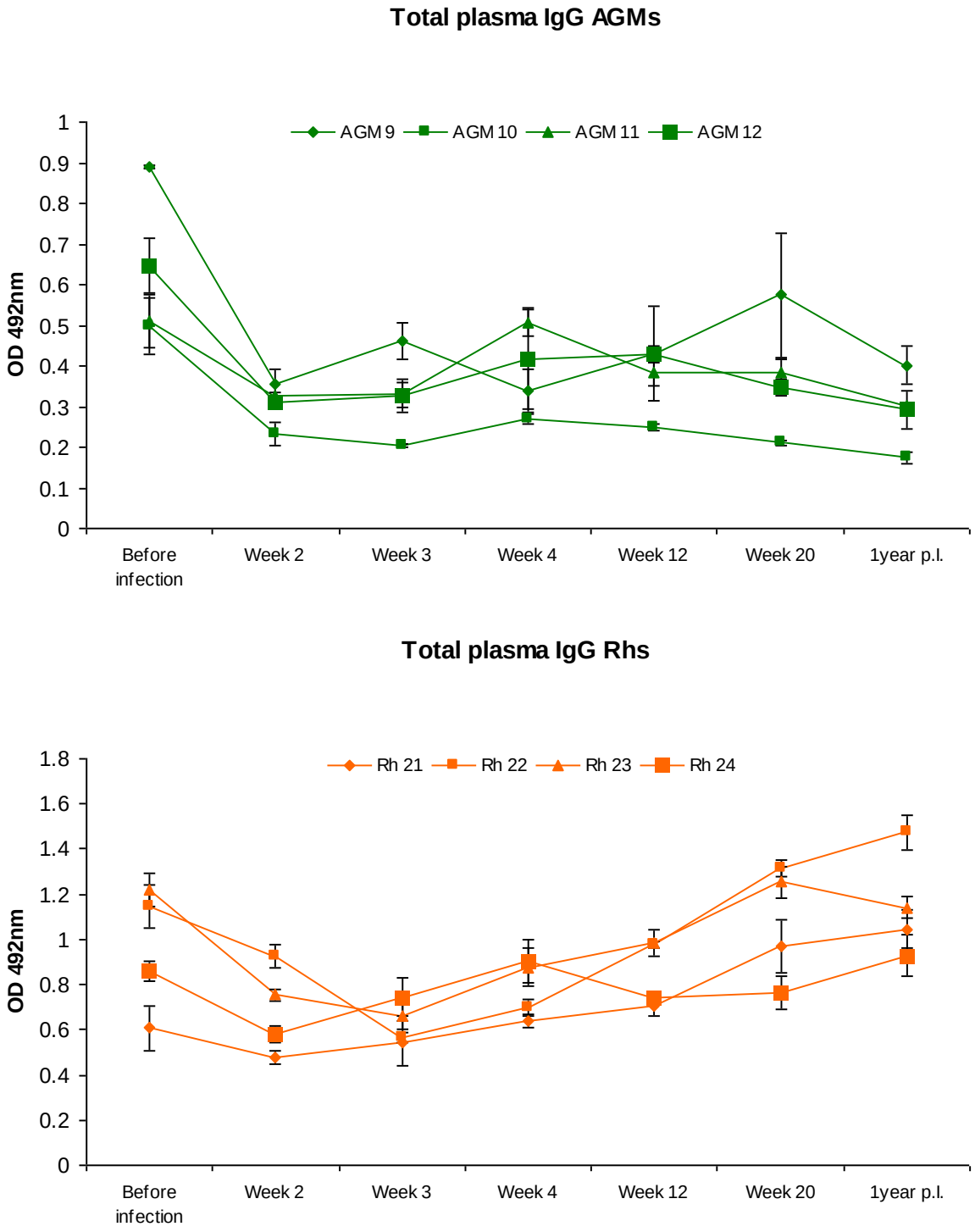

Figure 3.8.1b: Comparison by ELISA of total IgG in the plasma of macaques and AGMs taken before and at weeks 2, 3, 4, 12, 20 and one year after infection with SIVmac and SIVagm, respectively. Plasma diluted 1:10,000 was left to dry overnight and was detected using an anti-human IgG antibody.

In all four AGMs, the total IgG levels dropped as early as two weeks p.i. and did not rebound, remaining below baseline levels for up to one year after infection. In contrast, total IgG levels in rhesus macaques, despite an initial drop at 2-3 weeks p.i., rebounded and continually increased thereafter to levels near or exceeding baseline. This can best be seen in figure 3.8.1c that shows the trend for the development of total IgG upon SIV infection.

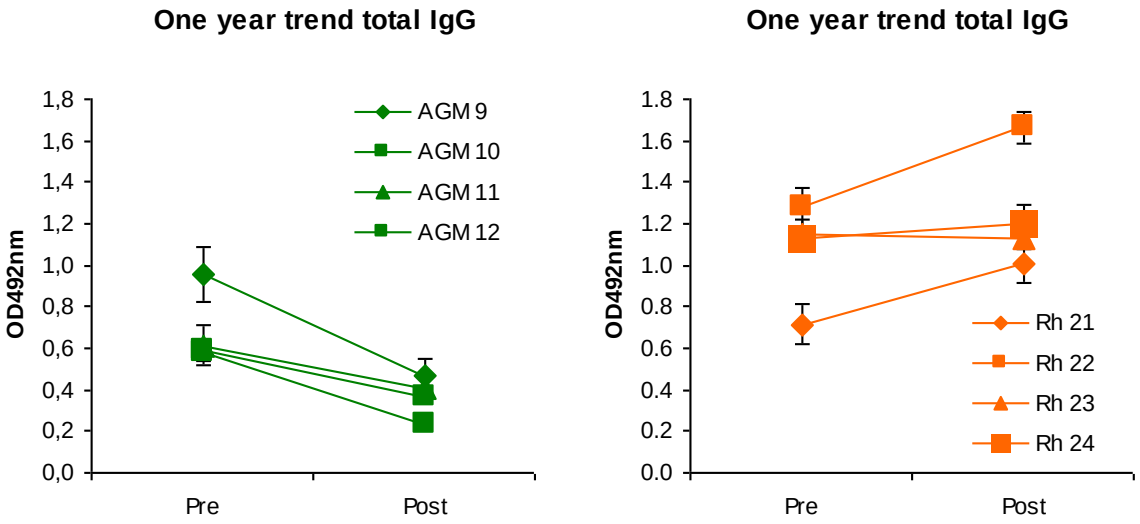

Figure 3.8.1c: Comparison of total IgG in the plasma of AGMs and macaques before and one year after infection with SIVagm or SIVmac, respectively.

3.8.2 Quantification of CD20$^+$ B-cells in AGMs and rhesus macaque PBMCs

The ELISAs for total IgG quantification in plasma repeatedly resulted in lower OD492nm values for AGMs compared to rhesus samples. Thus, it was investigated whether AGMs possess less B-cells than rhesus macaques in general (figure 3.8.2a). Indeed, the frequencies of CD20$^+$ (memory) B-cells were lower in AGMs (5.09-8.15%) compared with rhesus (11.0-27.9%).

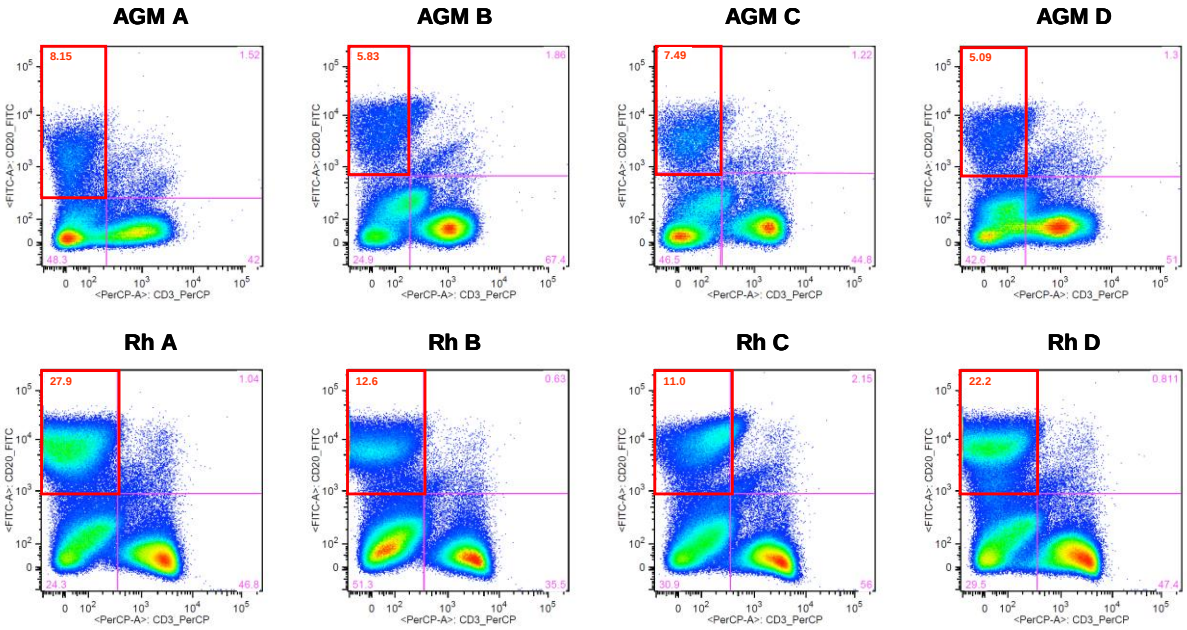

Figure 3.8.2: Quantification of CD20+ B-cells in PBMCs of 4 AGMs (upper row) and 4 rhesus macaques (lower row) with FACS. Flow cytometric analysis was performed with an LSRII flow cytometer on freshly isolated cells from SIV negative AGMs and Rhs. Cells were stained anti-CD20 FITC (2H7;BD) anti-CD3 PerCP (FN18;Biosource). $CD20^{+}$ B-cells were defined as $CD20^{+}$ $CD3^{-}$.

4 Discussion

4.1 The AG3.0 antibody

In our research group, it was shown that even though AGMs develop very high titers of antibodies against whole virus lysate (mainly containing Env proteins) of SIVagm, no antibodies against the viral Gag protein could be detected [180]. It further became evident that in many other natural host systems, the antibody response to Gag was also either weak or absent. It was therefore proposed that natural hosts for SIV had developed a form of selective tolerance to Gag proteins and that this could protect them from the development of AIDS by avoiding the trapping of immune complexes in their lymph nodes, which would lead to their destruction. In the previous studies involving immunisation of AGMs with SIVagm Gag or DNA coding for Gag protein (Holzammer, in preparation; Siegismund et al., submitted), it was not possible to determine whether the down-regulation of specific antibodies upon infection was protein-specific, virus-specific or systemic. The fact that infected AGMs developed high-titre antibodies to the Env glycoprotein despite losing the responses to Gag suggested that the lack of Gag-response is protein-specific. Whatever the mechanism, it was not possible to induce by immunisation the prolonged, high levels of Gag-specific antibodies concurrently with infection needed to test the hypothesis that antibody/Gag complexes contribute to disease in heterologous hosts.

To overcome this problem, the initial goal of this project was the cloning of a recombinant anti-Gag antibody gene into an adeno-associated virus (AAV) vector, in order to artificially produce sustained high anti-Gag antibody titers in African green monkeys prior to and after SIVagm infection. The recombinant AAVs would facilitate the production and secretion of a mouse/AGM hybrid antibody molecule (comprising the AG3.0 Fab fragment fused to the Fc-region derived from AGMs) in AGM muscle cells, which would then distribute the antibodies to the circulatory system. The same approach had previously been successfully used to induce neutralising antibody protection against SHIV in rhesus macaques [201]. AG3.0 is specific for the SIVagm Gag protein but also reacts with members of the HIV-2 lineage (HIV-2, SIVmac), HIV-1 lineage, and SIVrcm, recognising a shared minimal epitope SPRTLNA (Sanders-Beer et al., submitted). In addition, AG3.0 reacts with the Gag precursors p55 and p38 and the Gag-Pol precursor in infected cell lysates. The SPRTLNA epitope is conserved

throughout the HIV-1 lineage including SIVrcm, the HIV-2, and the SIVagm lineage and is partially conserved throughout the other SIV lineages (SIVlho, SIVsyk, SIVcol, SIVdeb, SIVtal, SIVgsn), although it is completely absent in non-primate lentiviruses. Neither loss of antibody binding through the development of escape mutations nor cross-reactivity with proteins from other viruses was therefore considered to be likely. As a first step in the project, the CDR region of the AG3.0 antibody was successfully sequenced (fig. 3.1b), together with the Fc-region of an AGM IgG2 antibody (data not shown).

4.1.1 T-cell dependency of secondary B-cell responses to Gag

However, within the first year it became known that the secondary antibody response to SIV Gag proteins strongly depends on T-cell help, whereas the antibody response to (trimeric) SIV Env is T-cell independent [197]. Since it is known that in infected natural hosts for SIV, hyperactivation of T-cells does not occur [162], the lack of T-cell stimulation of Gag-specific B-cells seemed a more parsimonious explanation for the lack of Gag-specific but presence of Env-specific antibodies in these animals. Of note, in HIV-infection, a decline of Gag-specific antibody responses has also been observed in late-stage AIDS patients, whereas Env-specific antibody titers remain undiminished, and low levels of Gag-specific antibody titers in HIV-infection have been suggested as a surrogate marker for the loss of T-cell help [202]. Since immunisations with both SIVagm Gag protein (Holzammer, in preparation) and SIVagm Gag DNA (Siegismund et al., submitted) both induced anti-Gag antibodies in AGMs prior to infection with SIVagm, it had become clear that there is no state of pre-existing tolerance to this particular protein in these animals. It was in any case difficult to envisage a mechanism for selective tolerance to a viral protein with which an organism has not had previous contact. In contrast, a general mechanism for down-regulating B-cell responses by dampening T-cell help is a more likely explanation for the loss of anti-Gag antibodies upon SIVagm infection observed in previously immunised AGMs and is also consistent with the development of high antibody titres against Env. In this scenario, the absence of anti-Gag antibodies in SIVagm-infected AGMs is simply a 'bystander effect' rather than the reason for the lack of disease progression in these animals. However, it would still be essential to determine the exact biological mechanism leading to the (systemic) lack of B-cell activation preventing production of antibodies to T-cell dependent antigens.

4.1.2 The absence of Gag-specific antibodies in SIVagm-infected AGMs in not absolute

It had been hypothesised that the lack of anti-Gag antibodies in natural hosts such as AGMs was instrumental in preventing disease, a phenomenon that the previous SIV*gag* DNA immunisation study was designed to test. However, Gag-specific antibodies are not completely absent in SIVagm-infected AGMs or other natural hosts, although the titres are extremely low. Such antibodies can often be detected with highly sensitive methods such as radioimmunoprecipitation or western blot at very low plasma dilutions (C. Stahl-Henning, personal communication; [163, 203-205] reviewed in [183]) although usually not with ELISA, possibly due the lower sensitivity the latter. This observation supports the idea that the poor response to this protein is not due to immunological tolerance *per se*, as one would otherwise expect the response to be totally abrogated. It therefore seems likely that a blockage or dampening of the general mechanism for antibody production upon SIVagm-infection in AGMs exists. In order to address the nature of the mechanism possibly responsible for this phenomenon, it was decided to take a step back and obtain a broader overview of the immune activating and/or down-regulating processes in AGMs and rhesus macaques during SIV infection.

4.2 Plasma cytokine profiles in acutely SIV-infected AGMs and rhesus macaques

The availability of plasma panels from AGMs and rhesus macaques during acute SIV infection gave the opportunity for a comparative analysis of plasma cytokines and chemokines during the acute phase of exponential viral replication to be performed. The results of these studies revealed, in most cases, clear patterns and differences in cytokine/chemokine dynamics between the two species and/or immunisation groups.

4.2.1 Differences between species

The increase in viremia in acute SIV infection of rhesus macaques was associated with elevations in plasma levels of multiple cytokines and chemokines at week 1 p.i., including IFN-gamma, IL-1ra, IL-2, IL-4, IL-5, IL-6, TNF alpha, IL-15 and IFN alpha (the latter two with dramatic elevations). The pattern seen in rhesus was comparable to that seen in HIV-

infected individuals, especially for IL-15 and IFN alpha levels [114]. This is in stark contrast to AGMs, which often showed no or low cytokine/chemokine responses upon infection, with peak elevation levels occurring later than in the rhesus.

The role of IFN alpha in pathogenic HIV/SIV infection is controversial. PDC-derived IFN alpha has been shown to inhibit the replication of HIV in $CD4^+$ T-cells [206, 207], macrophages and monocytes [208], a phenomenon also shown *in vivo* using a model mimicking HIV-infection in humanised mice [209]. This is probably facilitated through IFN alpha-induced upregulation of APOBEC3G [210], an intrinsic factor that is able to induce the degradation of HIV DNA in the cytosol [211]. In addition, IFN alpha can inhibit apoptosis of both $CD4^+$ and $CD8^+$ T-cells [212], which in turn might prolong their survival during HIV/SIV infection. However, there are also clear detrimental effects of IFN alpha in HIV/SIV infection. During the acute phase, IFN alpha may lead to the recruitment of target cells into mucosal tissues and in chronic HIV/SIV infection, a persistent high level of IFN alpha is one driving factor for constant hyperimmune activation. The lack of systemic elevations in IFN alpha in the AGMs suggests that pDCs are not activated in these animals to the same extent as in rhesus macaques, consistent with reports in the literature [196, 213, 214], even though rapid and strong upregulation has been demonstrated in the lymphoid tissue of both sooty mangabeys and AGMs. Typically, this upregulation in the lymph nodes of natural hosts occurred within 24h of infection, but dropped back to baseline levels at 2 weeks p.i. [214, 215], whereas in the pathogenic system, levels remained consistently elevated during chronic infection.

Both IFN alpha and IL-15 lead to an increase in the number of NK-cells [216], which could be beneficial to the infected host. In fact, a profound expansion of NK-cell numbers has been observed during acute HIV infection prior to seroconversion [217]. NK-cells are able to modulate dendritic cell function, which can in turn have effects on overall adaptive immunity. Immature DCs in the periphery have been shown to be eliminated by NK cells, whilst mature DCs are spared [218, 219]. Hence, NK-cells can act as a means of quality control for those DCs that enter inductive sites of infection. However, NK-cells can also cause a reduction of uninfected $CD4^+$ T-cells that express NK-cell activating ligands and are therefore killed as a consequence of bystander activation [220, 221]. The high systemic IL-15 response seen in rhesus was completely absent in AGMs. One suggestion that arises from the lack of systemic IL-15 upregulation in AGMs during acute SIV-infection is that NK-cells are not necessary to

ensure survival of the infected host. Clearly, if NK-cell activation cannot be observed in the natural host, this mechanism could not account for the lack of pathogenicity. It is therefore unlikely that inducing responses that have not been selected for over millions of years of co-evolution of natural hosts and SIV will protect heterologous hosts such as rhesus and humans from disease progression. The detrimental effects of high IL-15 (and IFN alpha) levels in rhesus during the viral ramp-up phase are therefore probably overriding the restricting effects they might have on early virus replication.

IFN beta is mainly induced by signalling pathways other than those induced by HIV/SIV infection. Influenza virus infection, for example, is "sensed" in the cytosol and not intraendosomally in pDCs (reviewed in [222]). However, IFN-beta levels were increased in most rhesus and, to a lesser extent, AGMs but with fluctuating dynamics. As there is an overlap in the function of transcription factors induced by HIV/SIV and/or cytosol-detected viruses such as influenza (IRF-7 and IRF-3, respectively), the upregulations in IFN-beta levels are likely to be a bystander effect of the IFN alpha response in these animals.

Plasma levels of TNF alpha, which is also released by pDCs upon TLR ligation, were not as clearly distinct between the two species as IFN alpha and IL-15. However, the upregulation appeared later in AGMs than in rhesus macaques (week 2 or 3 vs. week 1 p.i.), and fewer AGMs responded. However, it has been demonstrated that the *in vitro* TNF alpha responses of sooty mangabey pDCs to TLR 7/9 stimulation does not significantly differ from that seen in rhesus macaques [198], and TNF alpha is also secreted by activated myeloid DCs (mDCs), which also express TLRs 7 and 9 [223]. TNF alpha has been shown to inhibit the generation of pDCs from hematopoietic progenitors [224] and might therefore have in part accounted for the profound decrease of systemic IFN alpha levels at week 2 p.i. in the rhesus. Even though the latter effect is anti-inflammatory, TNF alpha also acts as a proinflammatory cytokine that up-regulates the production of acute-phase proteins that can inhibit the replication of HIV [225]. This same function accounts for IL-6 (reviewed in [226]), which was only upregulated in rhesus but not AGMs.

Slow elevations of IL-12 can be observed during acute HIV infection and this is consistent with our results in the rhesus and in a number of the AGMs. IL-12 is mainly secreted by activated macrophages and monocytes and can be counteracted by IFN alpha. This could

explain the apparent decrease of IL-12 seen at week 1 p.i. in many rhesus macaques when IFN alpha levels peaked in these animals.

MIP-1alpha and MIP-1beta both possess anti-HIV activity [227] but are only upregulated in a small proportion of HIV-infected subjects. This is consistent with our findings in the rhesus with more showing elevations of MIP-1beta than MIP-1alpha. Even though higher levels of MIP-1beta were also seen in some AGMs, these were lower than in the rhesus, indicating lower levels of immune activation.

No information could be obtained about IL-10 levels, due to a lack of assay specificity and limited amounts of plasma (an attempt to measure levels by ELISA failed, data not shown). However, in humans there is a late increase of IL-10 levels in acute HIV infection that peaks at around the same time as plasma virus load [114]. In AGMs, it was found that FoxP3, TGF-beta and IL-10 are upregulated as early as 24h p.i., which indicates a rapid induction of regulatory T-cells by DCs [184].

IL1Ra is secreted by various types of cells including monocytes, macrophages, neutrophils, mast cells, epithelial cells, skin keratinocytes, stromal cells, astrocytes and adipocytes (reviewed in [228]), and binds to IL-1 receptors, thereby competitively antagonising the inflammatory effects of IL-1 alpha and IL-1 beta [229]. IL-1Ra could therefore act as a preventative mechanism against excessive inflammatory responses induced by IL-1. In a healthy population, IL-1/IL-1Ra ratios have been shown to be close to 1 [230], whereas serum levels of IL-1Ra are elevated in a variety of autoimmune diseases. In contrast, inflamed tissues usually have elevated levels of IL-1. An increase in IL-1Ra corresponds to a delayed response upon IL-1 production. Therefore, even though IL-1Ra acts as an anti-inflammatory cytokine, the elevated levels in plasma of the rhesus probably typify the inflammatory processes occurring in acutely SIV-infected lymphoid tissue.

The main cells expressing CD40L are activated T-cells. CD40-CD40L interaction is pivotal for T-cell dependent B-cell activation (reviewed in [231, 232]), as an activation of CD40 on immature B-cells leads to proliferation, differentiation and IgG production. Conversely, CD40 activation does prevent the terminal differentiation of activated, mature B-cells into plasma cells [233]. In addition, CD40 stimulation on antigen-presenting cells enhances their ability to activate naïve T-cells [234]. In its soluble form, CD40L might act in the same manner on B-

and T-cells as the cell-bound form. In addition, plasma elevations of sCD40L are known for their association with an increased risk for cardiovascular disease [235]. In our study, baseline sCD40L levels varied in both species, although on average they were higher in the AGMs than in the rhesus macaques. This, together with the fact that levels were higher in the immunised compared to the control group in both species suggests a role of sCD40L in developing immune responses to an antigen. However, beyond this point, the inter- and intra-species variations in sCD40L were too large to identify a clear pattern. It is nevertheless interesting that elevated sCD40L was one of the very few changes that occurred at the same time in AGMs and rhesus macaques.

4.2.2 Differences between immunisation groups

In some cases, plasma cytokine responses were absent in those animals that had not been immunised, or the elevations were not as profound as in the immunised animals of the same species. There was a large increase in IL-4 in the immunised rhesus, which is probably due to an anamnestic response to the antigen and the activation of B-cells in these animals. In contrast, no elevations of IL-4 were seen in the control rhesus. In AGMs, the response was completely absent, an observation that is consistent with the lack or transient nature of the humoural response to the antigen in both immunised and control animals.

IL-18 levels showed a similar pattern, with no detectable increases in AGMs, but strong responses in the immunised rhesus. Of the control rhesus, only one (Rh22) showed a slight increase at week 1 p.i. while all others remained at baseline. In the presence of IL-12, IL-18 induces the production of IFN-gamma in T-helper type 1 (Th1) cells, NK-cells, B-cells and DCs [236-238]. However, in this study, the changes in IFN-gamma levels in rhesus were comparable (with the exception of very high peaks of rhesus 14 and 16 of the SIVagmGag DNA immunisation group at week 1 p.i.), but this was also true for levels of IL-12. IL-18 has been demonstrated to stimulate the replication of HIV-1 [239]. Also, it is one of the major correlates for coronary heart disease in HIV-infection [240].

A tendency for lower responses in control animals was also observed for MIP-1beta in both rhesus and AGMs. In the latter, levels of sCD40L were also lower in control animals than in the immunised groups, indicating that it is indeed possible to prime the AGM immune system for SIVagm to a certain extent.

Taken together, the plasma cytokine data clearly demonstrate that rhesus macaques undergo stronger immune activation during acute SIV infection than do AGMs. Indeed, patterns of systemic cytokine levels in the former are in agreement with those found in humans during acute HIV infection. However, in the SIVgagDNA immunisation study, plasma samples were taken on only a weekly basis. As cytokine levels are very dynamic, peak levels of some cytokines may therefore have been missed. For high-resolution immune activation profiles it would be necessary to take samples at shorter intervals.

It should be noted that the tendency of cytokine and chemokine responses to be higher in immunised than control animals during acute SIV infection should be taken into account for vaccine studies. A non-protecting vaccine could prime recipients for higher levels of activating cytokines, which in turn could activate more target cells and therefore result in faster progression to disease. This is supported by the results of a recent study comparing immune activation profiles during (mostly) transient, apathogenic SHIVsf162P3 infection and persistent, pathogenic SIVmac251 infection in rhesus [241]. Levels of activated, proliferating T-cells and proinflammatory, Th-1 and Th-2 type cytokines as well as most chemokines were consistently higher in those rhesus infected with the pathogenic SIVmac251 compared to those animals infected with SHIVsf162P3. During infection with the latter, although a high peak viremia was observed at week 2 post infection, this was "cleared" to undetectable levels 70 days p.i. This was in contrast to infection with SIVmac251 infection where peak virus loads and set-point viremia resembled those found in HIV infection of humans. The authors suggested that an early constrained immune activation and proliferation during acute SIV/HIV infection might lead to a reduced destruction of lymphocytes, thereby enabling the immune system to mount an effective immune response to clear the infection. However, if this is indeed the case, there must be a very fine balance between insufficient and excessive immune activation, as the levels of proinflammatory markers detected in the AGMs in our study were even lower than those observed in the rhesus infected with SHIVsf162P3 (animals that mostly controlled the infection after the acute phase). The AGMs, however, despite having even lower levels of immune activation, had set-point virus loads comparable to or exceeding those in SIVmac infection of macaques (figure 3.2, Siegismund et al., submitted). The acute-phase immune activation in AGMs might simply be too weak to initiate responses able to clear SIVagm infection.

It would also be of interest to compare immune activation during acute SIV infection of natural and heterologous hosts with those of other viral infections in these animals, as has been done for HIV, HBV and HCV in humans [114]. So far, it remains unclear whether the lack of chronic immune activation in infected AGMs is a specific hallmark of SIV infection.

4.3 Development of realtime-PCR assays for the detection of biomarker expression levels

Realtime PCR assays were developed for the determination of the levels mRNA expression for 32 biomarkers in chronically SIV-infected AGMs and rhesus macaques. Although both the rhesus and AGM mRNA sequences were already available for four of the biomarkers, the AGM mRNA sequences for the remaining 28 were unknown and had to be determined beforehand. Partial cDNAs of these biomarkers were successfully sequenced. Based on these sequences, primers that were simultaneously compatible with both AGM and rhesus cDNAs were designed for use in the realtime PCR. Using the same set of primers both species also (probably) ensured that the same splice variants were amplified. Although it cannot be excluded that some unknown variants were missed, primers were designed to amplify all known splice variants of each biomarker mRNA wherever possible. In addition, by aligning the AGM and rhesus cDNA sequences with the human genomic sequence for each biomarker, the primers were designed to bind in different (hypothetical) exons, with (hypothetical) intron sequences of sufficient length to exclude amplification of genomic DNA under the conditions used (i.e. a short amplification time for the ≤300bp fragments). This bypasses the need to eliminate potential DNA contamination in the RNA extractions, which can reduce the quality of the extracted RNA. In addition, the procedures for removing DNA contamination in RNA extractions (usually via DNA digesting enzymes or DNA elimination columns) are expensive.

A further feature for the design of the realtime PCR primers was to place them at distances of 140-300bp apart on the mRNA. This allowed the same elongation time to be used for amplification of all biomarker cDNAs, which in turn facilitated the running of all assays simultaneously in the realtime cycler using the same program. One crucial prerequisite for this was to ensure that the efficiency of the realtime PCR was the same for assays run with either AGM or rhesus cDNA as template and this was achieved with PCR efficiencies of 95-100% and very low deviations between the two species (0.02-0.5%).

There are two common methods used for the detection of amplified PCR products in realtime PCR. The first uses DNA-based specific probes designed to bind between the forward and reverse primers and that carry a fluorescent label and a quencher at opposite ends. During amplification of the DNA template, the hybridised probes are degraded by the exonuclease activity of the DNA polymerase, thereby releasing the fluorescent label from the proximity of the quencher. One advantage of using such probes is their specificity, which, to a great extent, excludes false positive signals. Major disadvantages however, include the relatively high costs of PCR probes and the fact that their requirement for specificity renders assays with many different targets less practical.

The second method for detecting amplified DNA with realtime PCR is to use Eva green or SYBR green. These dyes bind to double-stranded DNA and, in doing so, emit green fluorescent light. The compounds are available at a fraction of the cost of specific probes and, due to their lack of specificity, can be used for any given PCR. For these reasons, this system was selected for the detection of the amplified biomarker cDNAs. However, the lack of specificity does increase the risk of false positive resulting from background fluorescence created by the amplification of unspecific products or primer dimers. This can be counteracted by using annealing temperatures as high as possible in the PCR and by careful primer design.

The primers for biomarker mRNA detection were designed to have no more than three base pairs overlapping at the 3' end, resulting in a very low Tm for potential primer dimers. This reduces the risk of amplifying primer dimers at higher temperatures and, if primer dimers do form, the increase in fluorescent signal from the dimers will be linear in contrast to the exponential increase of amplified biomarker products. Therefore, given a sufficient quantity of template at the start of the reaction, the threshold-crossing ct value from amplified primer dimers will appear much later than the product of the amplified biomarker mRNA. In addition, if a negative control sample gives a detectable ct value but the dissociation curve in the negative control sample is in primer dimer range without any peak in the product range, the sample can be considered negative. Finally, in the very unlikely event of genomic DNA being amplified, the very long products generated would give shifted dissociation curves.

Taken together, careful primer design taking into account the parameters mentioned above can yield significant benefits in terms of costs and practicability.

4.4 Biomarker expression levels in chronically SIV-infected AGMs and rhesus macaques

The expression levels of 32 biomarker mRNAs in PBMCs from chronically SIV-infected AGMs and rhesus macaques were compared using the newly developed realtime RT-PCR assays. The levels and functions of those biomarkers that were also detected at the protein level in the plasma of acutely SIV-infected AGMs and rhesus macaques have been discussed earlier. Of these, no differences in the mRNA expression levels were detected for IFN alpha, IFN gamma, TNF alpha, IL-4, IL-1Ra, GM-CSF and MIP-1beta. For MIP-1 alpha and IL-15, a tendency for higher levels of expression in rhesus was observed, but this could not be confirmed due to the limited sample numbers.

IL-7 is an important factor for the regulation of peripheral T-cell homeostasis ([242], reviewed in [243]) and is produced primarily in non-hematopoietic stromal and epithelial cells throughout most organs including the brain. It is also produced by dendritic cells and macrophages, albeit in lower amounts. Plasma IL-7 levels have also been reported to be elevated during HIV-induced lymphopoenia [244-246]. Although recovering $CD4^+$ cell levels are associated with a further decline in IL-7 levels, the mechanism this is still unknown. IL-7 is essential for B-cell development at an early stage, but in mature B-cells the receptor for IL-7 is silenced. In this study, IL-7 expression levels in PBMCs of chronically SIV-infected AGMs and rhesus did not significantly differ from each other. However, since the main source for IL-7 are cells other than PBMCs, this observation may not be of particular significance.

IL-16 was originally termed lymphocyte chemoattractant factor by Center et al (1982) because if its chemoattractant effect on $CD4^+$ lymphocytes and monocytes. Indeed, a direct physical IL-16-CD4 interaction was later demonstrated [247]. The molecule is synthesised as a 631 amino acid precursor [248] in a vast number of cell types, including $CD4^+$ and $CD8^+$ T-cells, mast cells, eosinophils, dendritic and epithelial cells and fibroblasts, but trimeric, bioactive IL-16 has only so far been detected in $CD8^+$ T-cells (reviewed in [249]), suggesting that a large part of bioactive IL-16 is formed extracellular. IL-16 possesses anti-HIV activity [250-253], but although one might expect a mechanism of receptor blockade via its interaction with CD4, it does not directly inhibit viral entry into human T-cells. Instead, there is evidence for a restriction of HIV transcription by IL-16 [118-120]. In addition, IL-16 is able to prime mature memory $CD4^+$ T-cells in the peripheral blood to proliferate in response to IL-

2 [254]. Due to these effects of IL-16, it is not surprising that there is a negative correlation between IL-16 levels and disease progression in HIV-infected individuals [255, 256] and consistently high IL-16 levels have been reported in long-term nonprogressors [257]. In our study, there were no differences in the IL-16 expression of PBMCs from chronically SIV-infected AGMs and rhesus, even though the nonprogressor phenotype of the AGMs might have suggested higher levels in these animals. Conversely, lower levels of IL-16 mRNA in the AGMs might have indicated a possible mechanism by which T-helper cell-mediated activation of B-cells is dampened. However, since the bioactivity of IL-16 depends on the formation of a trimeric structure, it cannot be concluded that mRNA quantity directly relates to protein function. The role played by IL-16 in the different states of immune activation and B-cell responses to SIV-infection in AGMs and rhesus macaques therefore remains unclear.

IL-22 is a cytokine of the IL-10 family and, like IL-17, is a key regulator of homeostasis and epithelial barrier function. It is secreted by a broad variety of T cells and other leukocytes (reviewed in [258]) and binds to a heterodimer of the IL-10 receptor beta chain and the IL-22 receptor, the latter of which, with the exception of monocyte-derived macrophages [259], is exclusively expressed on tissue cells [260]. The impact of IL-22 on epithelial cells depends on the local microenvironment. In the presence of TNF alpha, IFN gamma and/or IL-17, a proinflammatory immune reaction is initiated, whereas IL-22 alone has an anti-inflammatory effect [261, 262]. However, the most important function of IL-22 in the context of HIV/SIV infection is the induction of innate immune responses in the epithelium that are directed against extracellular pathogens. It induces the secretion of antimicrobial peptides and re-epithelialisation and is an enhancing factor for migration and proliferation of epithelial cells [263]. Therefore, by enhancing the defence mechanisms at mucosal barriers such as the gut and maintaining or restoring their physical integrity, hyperimmune activating events such as microbial translocation could be prevented by the action of IL-22. The AGMs and rhesus PBMC used in this study showed differences in neither IL-22 expression levels nor TNF alpha or IFN gamma expression levels. However, IL-22^{+} leukocytes are more often found in peripheral tissue rather than in circulation [262].

Chemokines are chemoattractant cytokines that can be classified into four subfamilies according to their number and location of cysteine residues at the N-terminal end (CXC, CC, CX3C and C; Rollins, 1997). Their main function is the regulation of cell trafficking by recruiting cells expressing the respective receptors, such as lymphocytes, monocytes and

neutrophils, which migrate along a gradient towards the high chemokine concentrations at their source (reviewed in [264]). The chemokines whose mRNA expression levels were investigated in this study (systematic names are given in brackets) are MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10) and IL-8 (CXCL8). All four have implications in HIV/SIV pathogenesis. MCP-1 (reviewed in [265]) is mainly expressed by monocytes and macrophages and attracts cells expressing its receptor CCR2, such as monocytes themselves and memory T-cells [266, 267]. RANTES is expressed by NK-cells [268] and activated T-cells and attracts cells expressing CCR1, CCR3 and CCR5. A variety of immune and non-immune cells express IL-8 (reviewed in [269]) which binds to CXCR1 and CXCR2 on neutrophils. The ligands for IP-10 are CXCR3-A and –B, and it is expressed by variety of cells, including monocytes, endothelial cells, keratinocytes, and fibroblasts, being induced mainly by IFN-gamma. This molecule can activate the migration of polyclonal human NK-cells and activate cytotoxicity in resting NK-cells (reviewed in [270]). There is a positive correlation between pVL and plasma MCP-1 levels [271, 272] and elevated levels of MCP-1, IP-10 and IL-8 have been found in the cerebrospinal fluids of humans and rhesus with HIV/SIV-related neurological disorders [273-277]. High MCP-1 levels have also been associated with atherosclerosis and cardiovascular disease [278, 279], which only recently is being recognised as problematic side effect of long-term HIV-infection despite antiretroviral therapy (reviewed in [240]). In contrast, MCP-1, RANTES and IL-8 have been suggested to be inhibitors of HIV-1 infection by competitively inhibiting viral attachment through the coreceptors CCR2 and CCR5 [25, 280]; though the effect of MCP-1 remains controversial [281]. MCP-1 also plays a role in the control of Th2 polarisation [282], whereas RANTES, interacting with CCR3 on T-cells, triggers Th1 responses (reviewed in [283]). This, and differing expression levels in SIV-infected rhesus compared to AGMs could suggest a mechanism for the lack of Th2 (i.e. B-cell) responses in the latter, for example by lower (MCP-1) or higher (RANTES) activation of cell types expressing these chemokines. However, in this study, expression levels of all chemokines were comparable between AGMs and rhesus.

IL-1 alpha is an intracellular acting cytokine of the epidermis that activates fibroblasts and VEGF is an important growth factor for vascular tissue. Together with the proinflammatory cytokine IL-18, they are involved in cardiovascular inflammation and elevated levels are major prediction factors for coronary heart disease in both HIV-negative and –infected

individuals (reviewed in [240]). Levels of these biomarkers were also not significantly different between rhesus and AGM PBMCs in this study.

For both of the 'Th1-cytokines' that were measured (IFN gamma and TNF alpha), no significant differences in expression levels was observed between the two species. However, it has been reported that $CD8^+$ T-cell responses against SIV are lower in AGMs than in rhesus macaques, at least for some antigens ([177, 178] and Siegismund et al., submitted).

IL-10 is an immunoregulatory cytokine that can have both immunosuppressive and stimulatory effects (reviewed in [284, 285]). It can be expressed by various cell types, including T- and B-cells, dendritic cells and macrophages and can suppress cytokine production by and proliferation of $CD4^+$ and $CD8^+$ T-cells. Genetic polymorphisms that are associated with increased IL-10 expression are associated with increased severity of chronic viral infections such as HBV and HCV [286-289] and slower progression to AIDS has been observed in HIV-infected individuals carrying polymorphism that reduce IL-10 expression [290]. In contrast, IL-10 can have a stimulatory effect on NK-and B-cells, leading to their proliferation [291, 292]. Furthermore IL-10 can enhance B-cell survival [293]. For this reason, IL-10 is often considered to be a cytokine of the Th2 response. In this study, levels of IL-10 expression in PBMCs of chronically SIV-infected rhesus were significantly increased compared to PBMCs of chronically SIV-infected AGMs. This is consistent with the lack of hyperimmune activation in natural hosts for SIV and could be one reason for the higher levels of plasma IgG observed in the rhesus of the SIVgagDNA immunisation study.

Another cytokine involved in shaping the immune response towards activating B-cells is IL-13, which is secreted by Th2 cells (reviewed in [294]). Although higher expression levels of IL-13 in the rhesus might therefore have been expected, no significant difference was seen between chronically SIV-infected AGMs and rhesus in this study.

Interestingly, levels of IL-6 were significantly increased in the SIV-infected AGMs compared with the rhesus. IL-6 acts as a proinflammatory cytokine with functional pleiotropy, as it activates diverse proliferative, differentiative and activation events on T-cells, B-cells, astrocytes and hepatocytes (reviewed in [226]). In addition to activating B-cells, it has also been shown to up-regulate the production of HIV-1 in monocytes [295]. It is therefore somewhat surprising that levels of IL-6 were up-regulated in PBMCs of SIV-infected AGMs,

whose non-pathogenic infection course is distinguished from that of heterologous hosts by an absence of hyperimmune activation.

FoxP3 is a marker expressed mainly on regulatory T-cells, which is why higher expression levels in PBMCs of SIV-infected AGMs could have indicated a mechanism by which T-helper cell responses are dampened in these animals. Indeed, a very early upregulation within 24h of SIV-infection in AGMs but not rhesus macaques has been reported [184]. In contrast, this could not be observed in acute SIV-infection of sooty mangabeys [191], although it is not necessarily the case that all natural hosts for SIV have developed the same mechanisms for avoiding T-cell hyperactivation and disease. However, no difference in FoxP3 expression could be observed in the PBMCs of chronically SIV-infected AGMs and rhesus macaques in this study.

PD1, expressed on T-cells, B-cells and monocytes, and its ligands PD-L1 and PD-L2 are best known as markers for the T-cell anergy that results in decreased cellular proliferation, cytotoxic function and cytokine secretion (reviewed in [296]) and are known as key players in the induction of immunological tolerance. PD-L1 is expressed on T- and B-cells, dendritic cells and macrophages, whereas PD-L2 is only expressed on dendritic cells, macrophages and bone marrow-derived cultured mast cells (reviewed in [297, 298]). "Exhausted" T-cells present high levels of PD-1 and receive a strong inhibitory signal that dampens T-cell receptor signalling upon ligation of cell surface PD-1 to PD-L1 or PD-L2 on antigen-presenting cells (reviewed in [299]). Elevated expression levels of PD-1 at the T-cell surface or increased expression levels of one or both of its ligands in AGMs could therefore induce a downregulation of T-cell (and hence B-cell) responses in these animals. In acute SIV-infection, a rapid increase in the expression of PD-1 has been demonstrated in the lymph nodes of SIV-infected sooty mangabeys but not rhesus macaques [191]. Conversely, PD-1 levels are high in rhesus lymph nodes during chronic infection (consistent with a response to long-term activation) but not in sooty mangabeys. That the PD-1 signalling system also affects B-cells and is a late player in the late humoral immune response has been recently demonstrated in mice [300]. An early activation of the PD-1 system in acutely SIV-infected sooty mangabeys and AGMs could therefore allow the initial humoral immune response to an antigen but prevent the establishment of long-term antibody-secreting plasma cells. In addition, it has been shown that an acute depletion of activated memory B-cells in rapidly progressing SIV-infected rhesus macaques involves the PD-1 pathway [301] and that an *in*

vivo blockade of PD-1 in these animals resulted in an enhancement of antibody-responses against both non-SIV and SIV antigens. However, in the same study, a depletion of activated memory B-cells in SIV-infected sooty mangabeys was not observed. It has been suggested that PD-L1 and PD-L2 have different functions in regulating Th1 and Th2 responses. PD-L1 might play a more general role in down-regulating activated ($CD4^+$ and $CD8^+$) T-cells in the periphery compared to PD-L2, which might have a more specific role on macrophages [302]. In this study, there were no differences in expression levels of PD1 or PD-L2 between SIV-infected AGMs and rhesus, but levels of PD-L1 expression were significantly higher in AGMs. This suggests an involvement of PD-L1 in SIV-infected AGMs in reducing T-cell activated B-cell responses to T-cell dependent antigens, such as Gag.

IRF-7 is a transcription factor for type-1 interferons (mainly IFN alpha) whose function will be discussed in more detail below. Levels of IRF-7 expression in PBMCs were significantly higher in the chronically SIV-infected rhesus compared to the AGMs, which is somewhat surprising given the fact that IFN alpha expression levels did not differ between the two species. However, since IRF-7 only acts upon activation, the degree of RNA expression does not necessarily reflect the quantity of active protein.

Taken together, the realtime RT-PCR data quantifying the expression levels of biomarkers in PBMCs from chronically SIV-infected AGMs and rhesus revealed significant differences in levels of IRF-7, IL-6, IL-10, and PD-L1. However, since pre-infection samples from these animals were not available, it cannot be unequivocally concluded that these differences were the result of SIV-infection only. Further analyses including uninfected control samples could shed light on this and would also facilitate a comparison of biomarker up- or down-regulations resulting from SIV infection. In addition, expression levels of biomarkers in PBMCs do not necessarily represent the activation status in other tissues. In the context of HIV/SIV-infection, lymphoid tissues, the gut mucosa and cardiovascular epithelium would be of particular interest. Finally, future studies should take into consideration the differences in distributions and quantities of cell types between AGMs and rhesus macaques, as analyses of expression levels in PBMCs tend to overlook this factor. Investigations of this nature could be facilitated by quantifying biomarker expression levels in homogenous lysates of previously isolated different cell populations.

4.5 Plasmacytoid dendritic cells and IFN alpha production in natural hosts for SIV

One observation that was published during the course of this thesis is the apparent inability of sooty mangabey pDCs to produce high levels of IFN alpha upon activation of intracellular TLRs [198]. PDCs can be found in various compartments of the body, such as the peripheral blood, thymus, inflamed skin, mucosa and lymph nodes. They are probably the first cells to encounter HIV/SIV after local entry of the virus. Because pDCs express Toll-like receptors (TLRs), they are a critical link between innate and adaptive immunity. Intracellular pathogens are sensed by TLRs and in the case of pDCs, viruses containing ssRNA (TLR7) or unmethylated CpG DNA (TLR9) are preferentially recognised, leading to the production of type 1 IFNs (see figures 4.5.2a and 4.5.2b). Even though the production of type 1 IFNs by pDCs can limit HIV/SIV replication [207, 303], there are also detrimental effects associated with pDC-infection in the pathogenic system. Transmission of HIV to T-cells can be facilitated by pDCs, and the released IFN alpha can induce apoptosis in uninfected T-cells, thereby contributing to $CD4^+$ T-cell loss [304-306]. The source of IFN alpha in the early acute phase of HIV infection is very likely to be pDCs. Since sooty mangabey pDCs have been shown to be less responsive to TLR7 and 9 activation compared to human or rhesus pDCs in vitro, it was postulated that the lower levels of type 1 IFNs in the natural system could protect the host from hyperimmune activation.

4.5.1 Interferon alpha responses of AGM and rhesus pDCs upon TLR7/9 stimulation

In support of the hypothesis that a reduced type I IFN production helps abrogate hyperimmune activation, AGM pDCs were found to produce lower levels of IFN alpha upon stimulation with HIV/SIV compared to those of rhesus macaques. IFN alpha secretion was blocked by adding inhibitors of TLR7 or by acidification of the endosome, confirming the need for TLR7 stimulation for IFN alpha secretion in HIV/SIV-stimulated pDCs. It was demonstrated that the same numbers of pDCs are activated in AGMs and rhesus macaques upon HIV/SIV infection in vitro, but that the effects of R-848 (a strong TLR7 agonist) or HIV/SIV on IFN alpha secretion appear to differ. Even though R-848 induced similar degrees of IFN alpha secretion (determined as cytokine activity) per spot in AGM and rhesus pDCs, there seemed to be a clear difference in the effect of SIV/HIV on TLR7 activation between the two species. One explanation could be the higher similarity between the SIVagm and

AGM genomes in terms of nucleotide composition compared to that of the SIVmac and the rhesus macaque genomes. It has been demonstrated that the more dissimilar the nucleotide composition of the ssRNA ligating to TLR7 is from that of the activated cells' genome, the higher are the resulting levels of secreted IFN alpha (N. Vabret, personal communication; reviewed [307]). Of note, Mandl et al. [198] also demonstrated significantly lower levels of IFN alpha production upon stimulation with R-848 in sooty mangabeys compared to rhesus macaques, an observation not repeated for AGMs in this study. Whether this was due to different assay specificities or an interspecies variation between sooty mangabey and AGM pDCs is unclear. However, there are also conflicting results concerning the secretion of IFN alpha from human pDCs upon R-848 stimulation [308, 309].

In the study by Mandl et al. [198], sequencing of many genes involved in TLR signalling revealed polymorphisms in IRF-7, a transcription factor involved in intracellular TLR signalling. Sooty mangabey IRF-7 was found to differ from that of humans or rhesus macaques in crucial domains. It was concluded that IRF-7 was a strong candidate as the factor responsible for the differences in IFN alpha secretion by sooty mangabey and rhesus pDCs.

4.5.2 Interferon regulatory factor 7

The mammalian IRF family comprises nine members (reviewed in [310]), each recognising a consensus DNA sequence known as the IFN-stimulated response element (ISRE) found in the promoters of genes encoding type I IFNs. IRFs reside in the cytosol and, following viral infection, undergo serine phosphorylation of their C-terminal region, allowing them to dimerise and translocate into the nucleus. IRF7 can form a homodimer or a heterodimer with IRF3, and these dimers have differential effects on the expression of the type I IFN gene-family members: IRF-7 homodimers target IFN alpha- and, to a lesser extent, –beta genes, whereas IRF-3 homodimers and IRF-7/IRF-3 heterodimers promote primarily the expression of IFN-beta and, only to a lesser extent, –alpha (figure 4.5.2a).

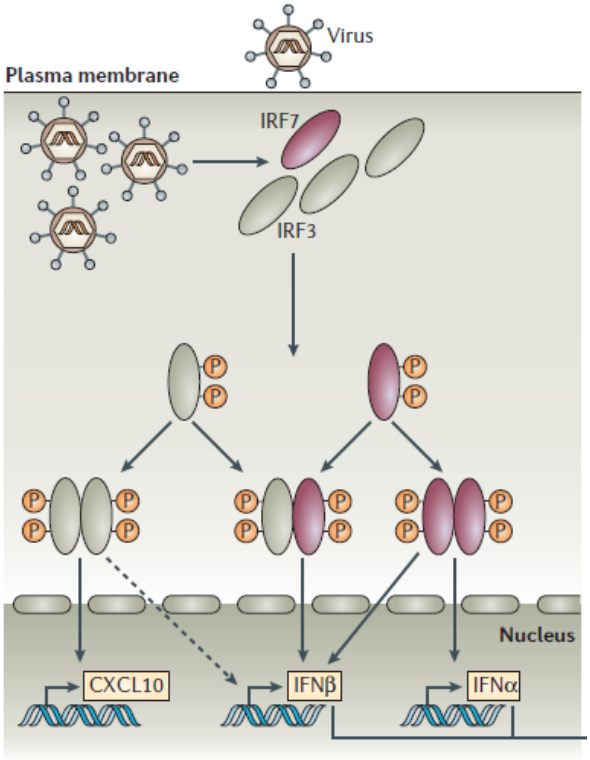

Figure 4.5.2a: Dimerisation of interferon regulatory factors 3 and 7 and induction of CXCL10, IFN beta and IFN alpha expression. From: [310].

There are two pathways for the induction of type-1 interferon expression: the virus- activated and the TLR-activated pathways (figure 4.5.2b). The virus-activated pathway refers to the recognition of viral components in the cytosol, which are sensed by pattern recognition receptors such as RIG-I and has been shown for infection of cells with influenza (reviewed in [222]). Briefly, dsRNA binds to RIG-I in the cytosol, leading to a conformational change in the protein. This results in binding to a complex of molecules on the mitochondrial membrane, which in turn leads downstream of a further signalling cascade, to the activation of the kinase TBK1. TBK1 phosphorylates IRF-3 and IRF-7 amino-terminal serine residues, leading to the formation of dimers. Once dimerised, the proteins are translocated into the nucleus, where they act as transcription factors for type-1 IFNs. The TLR-activated pathway for type-1 IFN expression is induced by the binding of agonists to their respective TLR in the endosome, such as CpG oligonucleotides (TLR9), ssRNA (TLR7/9) or dsRNA (TLR3). Inactive, unphosphorylated IRF7 is attached to MyD88, which is localised at the cytosolic part of the endosomal membrane. After ligation of an intra-endosomal TLR with its agonist, the MyD88-bound IRF7 is activated by a protein-kinase cascade, leading to the formation of IRF-7 dimers, their translocation into the nucleus and the initiation of type-1 IFN expression.

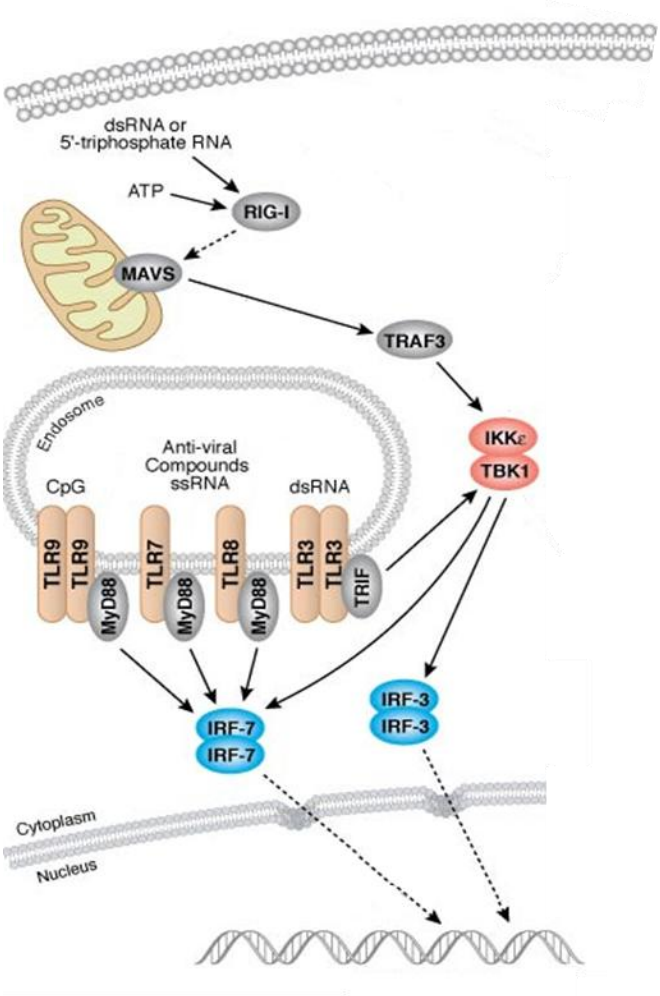

Figure 4.5.2b: Intracellular Toll-like receptor induced activation pathways. Adapted from:[311]

Activation of IRF-7 is facilitated by phosphorylation of serine residues in the C-terminal phosphorylation domain. This leads to the inactivation of the autoinhibitory domain of the protein which, in the inactivated stage of IRF-7, represses the transactivation domain. Once attached to the ISRE via the DNA-binding domain, the transactivation domain of (phosphorylated) IRF-7 serves as a protein interaction domain for the recruitment of further transcription factors for type-1 IFN genes [312, 313]. It is not yet known what the exact mechanism for IRF-7 phosphorylation is and it has been proposed that an unknown kinase might be involved in the process [314]. A schematic representation of the functional domains of IRF-7 is shown in figure 4.5.2c.

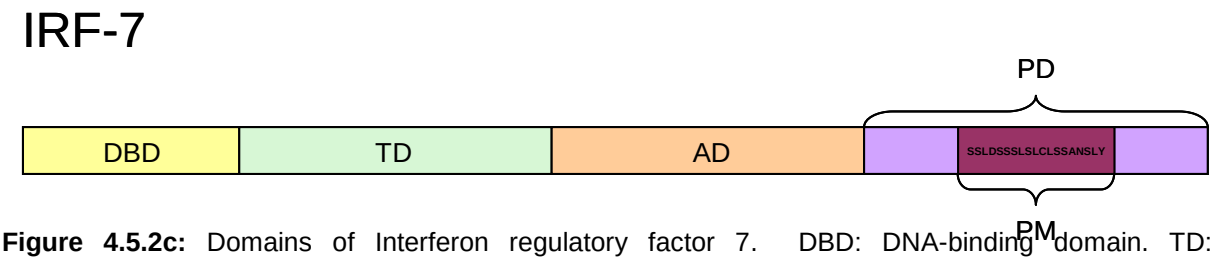

Figure 4.5.2c: Domains of Interferon regulatory factor 7. DBD: DNA-binding domain. TD: Transactivation domain. AD: Autoinhibitory domain. PD: phosphorylation domain. PM: Phosphorylation motif SSLDSSSLSLCLSSANSL.

4.5.3 Polymorphisms in the AGM IRF-7 mRNA sequence

In this study, the AGM IRF-7 gene was sequenced and compared to that of three other species (human, rhesus and sooty mangabey; figure 3.6) in an attempt to identify possible differences that could influence disease progression. Differences between the human and rhesus sequences were not taken into consideration, since both species are heterologous, disease susceptible hosts for SIV and HIV. There are six SNPs in the sooty mangabey sequence compared to the human and rhesus sequence, all located within the transactivation domain of IRF-7. Seven SNPs of the AGM sequence compared to human and rhesus could be identified (figure 3.6), five located within the transactivation domain of the protein, one in the N-terminal DNA binding domain and one in the autoinhibitory domain. Only one SNP that is not present in the heterologous hosts is the same in both natural hosts (sooty mangabeys and AGMs): a c→ t conversion that results in the incorporation of a valine instead of an arginine (amino acid 268). The fact that only one shared polymorphism was found in the natural hosts suggests a crucial role for the alteration in the apparently changed IRF-7 function. Changes in IFN alpha secretion have also recently been demonstrated in humans with a SNP in the IRF-7 gene (rs12272434) that was found in 20% of the individuals studied [309]. Individuals homozygous for this SNP showed significantly lower levels of IFN alpha-positive pDCs upon stimulation with iHIV compared with those individuals carrying the most common polymorphism. However, rs12272434 is located in the autoinhibitory domain (amino acid 441) and has not yet been detected in rhesus macaques, sooty mangabeys or AGMs. This could of course be due to the fact that IRF-7 sequences from non-human primates have not yet been determined in sufficient numbers, but it does indicate that there could be various ways in which IRF-7 function can be altered by SNPs. It cannot therefore be concluded that the arginine to valine conversion in sooty mangabeys is solely responsible for the dampened IFN alpha response in their pDCs. Indeed, little is known about the exact molecular mechanisms of action of IRF-7 functional domains, and whether or not the polymorphisms

found in the IRF-7 gene of sooty mangabeys and AGMs have any effect on ISRE binding or phosphorylation of IRF-7 or IFN alpha gene transcription cannot be predicted.

4.6 Innate activation of memory B-cells

If TLR-signalling in AGMs is indeed impaired, it is probably due to a general mechanism and not a unique property of pDCs. The fact that AGM pDCs show a lower responsiveness to TLR7/9 ligands than those of rhesus and humans strongly suggests that this feature is present in all cell types expressing TLR7 and 9 in these animals. One such cell type is the memory B-cell, which can be activated to differentiate into antibody-secreting plasma cells in two ways. Either a memory B-cell is activated by binding to its specific antigen and receiving a second stimulatory signal from an antigen-specific T-helper cell (reviewed in [315, 316]) or it can be activated by the innate immune system after binding of a TLR to its agonist (reviewed in [317]). By doing so, B-cells can be activated in the absence of their specific antigen. This mechanism of memory B-cell activation accounts for the often life-long maintenance of antibody titres against immunising agents [318]. In most cases, the activation of a TLR alone is sufficient to induce activation of memory B-cells without the requirement for any further stimulatory signal. The only exception to this is the activation of memory B-cells via TLR7/9, which is dependent on the presence of IFN alpha [319, 320].

4.6.1 Total IgG levels in SIV-infected AGMs and rhesus macaques

As it was suspected that the absence of anti-Gag antibodies in SIV-infected AGMs is a general rather than a specific phenomenon for SIV, it was hypothesised that this would also be reflected in the total amount of IgG present in plasma after infection. If the dampened T-cell activation and/or failure to induce memory B-cells via TLR7/9 in SIV-infected AGMs unspecifically leads to lower titres of antibodies directed against T-cell dependent antigens (such as Gag), levels against all T-cell dependent antigens should decrease. Since antibody responses against most antigens depend on T-cell help, the total IgG levels should be lower in AGMs following SIVagm infection.

Indeed, total IgG titres in AGMs appeared to decrease after SIVagm infection (figures 3.8.1a-3.8.1c), which was in contrast to the situation in rhesus macaques. AGMs showed lower IgG titres in general, which was reflected in generally lower OD492nm values in the assays

compared to rhesus. However, the frequency of total $CD20^+$ cells in AGM PBMCs was found to be about 5-8%, compared to the 12-27% in rhesus macaques (figure 3.8.2a). Of note, levels of circulating B-cells in sooty mangabeys have also been reported to be lower than in rhesus macaques [301]. The generally lower baseline total IgG titres may therefore reflect the overall lower B-cell counts in AGMs compared to the rhesus.

In all AGMs, there was a drop in total IgG upon SIVagm infection and levels remained below baseline even one year after infection (figures 3.8.1b and 3.8.1c). This supports the hypothesis of a generally lower B-cell response to T-cell dependent antigens upon SIVagm-infection in these animals. In rhesus however, total IgG titres either increased back to baseline levels as SIVmac infection progressed or increased to levels higher than before infection. Taken together, the data show a clear decrease of total IgG in AGMs and a trend of an increase of total IgG in rhesus over the course of SIV infection. The latter is possibly a mark of the hypergammaglobulinaemia that has also been reported in AIDS patients (reviewed in [321]).

To further confirm a drop in antibody titres against T-cell dependent antigens following SIVagm infection in AGMs, attempts were made to measure antibody levels that had been induced to other antigens prior to SIV-infection. The results of an ELISA measurement of IgG titres against simian foamy virus in the AGMs and rhesus were inconclusive (data not shown). However, since simian foamy virus is also a retrovirus, it would be more desirable to determine the change in IgG titre against a completely unrelated antigen. Thus, it would be interesting to further address this question in a future study in which AGMs and rhesus macaques are immunised with tetanus toxoid prior to SIV-infection, comparable to similar studies conducted in humans [202].

5 Conclusion and Perspective

The extremely low titres, but not absolute absence, of (T-cell dependent) anti-Gag antibodies in SIV-infected AGMs, despite concomitant high titres of (T-cell independent) anti-Env antibodies, suggest that one or more general mechanism(s) are involved in the phenomenon of dampened response. Very distinct differences in the immune activation profiles of natural (AGMs) and heterologous (rhesus) hosts during the acute phase of SIV infection could be identified in this study. First, rhesus macaques show distinctly higher levels of immune activation (determined as peak levels of proinflammatory cytokines) upon SIV-infection than AGMs. Second, there is a clear difference in the timing of peak cytokine elevations, with levels in the AGMs not only being lower, but also occurring about one week later than in rhesus macaques. The different timing in elevations might be a very important factor for the induction of 'tolerance' to SIV in AGMs. In evolutionary terms, it would be of benefit for tolerance to develop more slowly than the rapidly induced innate inflammatory responses to a pathogen. A rapidly induced tolerance of the "wrong" pathogen could be detrimental and unlikely to be reversible.

The development of realtime RT-PCRs for AGM and rhesus biomarker expression provided useful tools for characterising immune activation profiles at the mRNA level. In addition to the detection of biomarker expression levels in heterogeneous cell suspensions such as PBMCs, they have potential for use in further studies. First, testing larger numbers of chronically SIV-infected and uninfected AGMs and rhesus macaques will allow a more consolidated analysis and allow the up- and down-regulation of biomarkers upon SIV-infection to be better compared. Second, the assays could be used to measure the immune activation status in different tissues such as lymph nodes, gut mucosa and cardiovascular epithelium.

Gaining more insights into the processes that distinguish non-pathogenic from pathogenic SIV-infection is a crucial step towards identifying mechanisms responsible for the lack of disease progression in natural hosts. In this study, two possible candidates involved in dampening T helper cell activation and hence B-cell responses to T-cell dependent antigens (such as Gag) in AGMs have been identified: The PD1-PD-L1 induced anergy of T helper cells and the severely reduced capacity for innate memory B-cell activation by TLR7 activation, due to the diminished IFN alpha responses of AGM pDCs to SIV.

It is not yet known if the phenomenon of extremely low levels of anti-Gag antibodies and the reduction of total IgG levels upon SIV-infection of AGMs are mere bystander effects of the infection or do indeed have implications for pathogenesis (such as avoiding the trapping of immune complexes in lymph nodes). Investigating these and their correlations with the candidates mentioned above should be the subject of future studies.

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Appendix

AGM biomarker sequences

AGM FoxP3, partial cds:

CCTCCTCTTCCTNCCTTGAACCCTATGCCACCATCGCAGCTGCAGCTGCCCACACTGCCCCT
AGTCATGGTGGCACCCTCCGGGGCACGGCTGGGCCCCTTGCCCCACTTACAGGCACTCCTCC
AGGACAGGCCACATTTCATGCACCAGCTCTCAACGGTGGATGCCCACGCCCGGACCCCTGTG
CTGCAGGTGCACCCCCTGGAGAGCCCAGCCATGATCAGCCTCCCACCACCCACCACTGCCAC
TGGGGTCTTCTCCCTCAAGGCCCGGCCTGGCCTCCCACCTGGGATCAACGTGGCCAGCCTGG
AATGGGTGTCCAGGGAGCCAGCACTGCTCTGCACCTTCCCAAATCCTGGTGCACCCAGGAAG
GACAGCACCCTTTCGGCCATGCCCCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAA
GTGGCCCGGATGTGAGAAGGTCTTCGAAGAGCCAGAGGACTTCCTCAAGCACTGCCAAGCAG
ACCATCTTCTGGATGAGAAGGGCAGGGCACAATGTCTCCTCCAGAGAGAGATGGTACAGTCT
CTGGAGCAGCAGCTGTTGCTGGAGAAGGAGAAGCTGAGTGCTATGCAAGCCCACCTGGCTGG
GAAAATGGCACTGACCAAGGCTTCATCTGTGGCATCATCTGACAAGGGCTCCTGCTGCATTG
TAGCTGCTGGCAGCCAAGGCAGTGCCGTCCCAGCCTTGTCTGGCCCCCGGGAGGCCCCTGAC
AGCCTGTTTGCTGTGCGGAGGCACCTGTGGGGTAGCCATGGAAACAGCACATTCCCAGAGTT
CCTTCACAACATGGACTACTTCAAGTTCCACAATATGCGACCCCCTTTCACCTATGCCACGC
TCATCCGCTGGGCCATCCTGGAGGCTCCAGAGAAGCAGCGGACACTCAATGAGATCTACCAC
TGGTTCACACGCATGTTCGCCTTCTTCAGAAACCATCCTGCCACCTGGAAGAACGCCATCCG
CCACAACCTGAGCCTGCACAAGTGCTTTGTGCGGGTGGAGAGCGAGAAGGGGGCTGTGTGGA
CCGTGGATGAGTTGGAGTTCCGCAAGAAAAGG

Exons: 1-42, 43-146, 147-288, 289-374, 375-482, 483-567, 568-650, 651-799, 800-878, 879-979, 980-1086

AGM IFN-gamma, partial cds:

GATGAAATATACAAGTTATATCTTGGCTTTTCAGCTCTGCATTGTTTTGGGTTCTCTTGGCT
GTTACTGCCAGGACCCATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTTTAATGCAGGT
GATCCAGATGTAGCAGATAATGGAACTCTTTTCTTAGACATCTTGAGGAATTGGAAAGAGGA
GAGTGACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTTACTTCAAACTTTTTAAAAACT
TCAAAGATGACCAGAGGATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATTAATGTCAAG
TTTTTCAATAGCAACAAAAAGAAACGGGATGACTTCGAAAAGCTGACCAATTATTCGGTAAC
TGACTTGAATGTCCAACGCAAAGCAGTACATGAGCTCATCCAAGTGATGGCTGAACTGTCAC
CAGCAGCTAAAATAGGGAAGCGAAAAAGGAGTCAGATGTTTCGAGGTCGAAGAGCATCCCAG
T

Exons: 1-115, 116-182, 183-366, 367-497

AGM IFN-beta, partial cds:

GCCTAGATTCCTACAAAGAAGCAGCAGTTTTCAGTGTCAGAAGCTCCTGTGGCAATTGAATG
GAAGTCTTGAATATTGCCTCAAGGACAGGATGAACTTTGACATCCCTGAGGAAATTAAGCAG
CCGCAGCAGTTCCAGAAGGAGGACGCTGCATTGACCATCTATGAGATGCTCCAGAACATCTT

TGCTATTTTCAGACAAGATTTATCTAGCACTGGCTGGAATGAGACTATTGTGGAGAAACTCC
TTGCTAATGTCTATCATCAGATAGACCATCTGAAGACAATCCTAGAAGAAAAACTAGAAAAA
GAAGATTTCACCAGGGGAAAATTCATGAGCAGTCTGCACCTGAAAAGATACTATGGAAGGAT
TCTGCATTACTTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAA
TCCTCAGGAACTTTTTCTTCATTAACAAACTTACAGGTTACCTCCGAAACTGAAGATCTCCT
AGCCTGTGCCT

Exons: 1-507

AGM IL-1 alpha, partial cds:

CATTGACATCTGTCTCTGAATCAGAAATCCTTTCTGAAAAAAGCTATGACCCACTCCATGAA
GGCTGCATGGATCAGTCTGTGTCTCTGAGTATCTATGAAATCTCTAAAACATCCAAGCTGAC
CTTCAAGCAGAGCATGGTGGTAGTATCAACCAATGGGAAGGTTCTGAAGAAGAGACGGTTGA
GTTTAAGCCAGTCCATCACTGATAATAACCTGGAGGCCATCGCCAACAACTCAGAGGAAGAA
ATCATCAAGCCCAGGTCGGCACCTTTTAGCTTCCTAAGCAATGTGAAATACAACTTTATAAG
GATCATCAAACACGAATTCATCCTGAATGACGCCCTCAATCAAACTATAATTCGAGCCAATG
ATCAGTACCTCACGGCTGCTACAATACATAATTTGGATGAAGCAGTGAAATTTGACATGGGT
GCTTATGCGTCATCAAAGGACGATACTAAAGTTCCTGTGATTCTAAGAATCTCAAAAACTCA
ATTGTATCTGAGTGCCCAAGATGAAGACCAACCGGTGCTACTGAAGGAGATGCCTGAGATAC
CCAAAACCATCACAGGTAGTGAGACCAACTTCCTCTTCTTCT
GGGAAACTCATGGCACTAAGAACTACTTCGTATCAGTTGCCCATCCAAACTTGTTTATTGCC
ACAAAGCACGACAATTGGGTGTGCTTGGCAAAGGGGCTACCCTCCATCACTGACTTTCAGAT
ACTGGAAAACCAGGCGTAGA

Exons: 1-200, 201-370, 371-496, 497-700

AGM IL-10, partial cds:

AGAAGGCATGCACAGCTCAGCACTGCTCTGTTGCCTGGTCCTCCTGACTGGGGTGAGGGCCA
GCCCAGGCCAGGGCACTCAGTCTGAGAACAGCTGCACCCGCTTCCCAGGCAACCTGCCTCAC
ATGCTTCGAGACCTCCGAGATGCCTTCAGCAGAGTGAAGACTTTCTTTCAAATGAAGGATCA
GCTGGACAACATATTGTTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGGGTTGCC
AAGCCTTGTCTGAGATGATCCAGTTTTACCTGGAGGAGGTGATGCCCCAAGCTGAGAACCAC
GACCCAGACATCAAGGAGCATGTGAACTCCCTGGGGGAGAATCTGAAGACCCTCAGGCTGAG
GCTGCGGCGCTGTCATCGATTTCTTCCCTGTGAAAACAAGAGCAAGGCCGTGGAGCAGGTGA
AGAATGCCTTTAGTAAGCTCCAAGAGAAAGGTGTCTACAAAGCCATGAGTGAGTTTGACATC
TTCATCAACTACATAGAAGCCTACATGACAATGAAGATACAAAACTGAGACATCAGGGTGGC
GACTCTATAGACTCTAGGACATAAATTAGAGGTCTCCAAAATCAGATCCGGGGTTCTGGGAT
AGCTGACCCAGCCCCTTGAGAAA

Exons: 1-172, 173-230, 231-385, 386-449, 450-643

AGM IL-12 alpha, partial cds:

GTGGCCACCGCAGGCCCAGAAATGTTCCCGTGCCTTCACCACTCCCAAAACCTGCTGAAGGC
CGCCAGCAACACGCTTCAGAAGGCCAGACAAATTCTAGAATTTTACCCTTGTACTTCTGAAG
AGATTGATCATGAAGATATCACGAAAGATAAAACCAGCACAGTAGAGGCCTGTTTACCATTG
GAATTAATCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACTTCTTTCATAACTAATGGGAG

TTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAGTATTTATGAAG
ACTTGAAGATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGATGGATCCTAAG
AGGCAGATCTTTCTAGATCAAAACATACTGGGAGTTATTGATGAGCTGATGCAGGCCCTGAA
TTTCAACAATGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAACTA
AAATCAAGCTCTGCATACTTCTTCATG

Exons: 1-82, 83-193, 194-240, 241-281, 282-424, 425-523

AGM IL-12 beta, partial cds:

CCATCCCAGTCAAAAGAGTTTGGGAGGATGTTGGCCAGATACACCGTCACAAAGGAAGGCGA
GGGCTTTTAGCCATTCGCTCATGGTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGAT
ATTTAAAGGGCCAGAAAGAACCCAAAAATAAGACCTTTGTAAGATGCGAGAACAAAAATTAT
TCTGGACGTCTCGGCTGTTGGTGGCTGCCGACAATCAGTAATAATTTGACATTCAGTGTCAA
AAGCAGCAGAGGCTTTTTTAGCCCCCAAGGGGTGACGTGCGGAGTTGCTACACTCTTGCAGA
GAGGGTCAGAGGGGACAATAAGGAGTATGAGTACTCAGTGGAGCGCCAGGAGGACAGTGCCT
GCCCAGCCGCGGAGGAGAGGCTGCCCATTGAGGTCATGGTGGATGCCATTCACAAGCTGAAG
TATGAAAAATACACCAGCAGCTTTTTCATCAGGGACATCATCAAACCCGACCCACCCAAGAA
CTTGCAGCCGAAGCCATTAAAGAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACA
CCTGGAGTACTCCACATTCCTATTTCTCCCTGGCATTATGCATTCAGGTCCAGGGCAAGAGC
AAGAGAGAAAAGAAAGATAG

Exons: 1-140, 141-259, 260-474, 475-630,631-640

AGM IL-13, partial cds:

CGGTCATTGGCTCTCACTTGCCTCGGCGGCTTTGCCTCCCCAAGCCCTGTGCCTCCCTCCAC
AGCCCTCAAGGAGCTCATTGAGGAGCTGGTCAACATCACCCAGAACCAGAAGGCCCCGCTCT
GCAATGGCAGCATGGTGTGGAGCATCAACCTGACAGCTGGCGTGTACTGTGCAGCCCTGGAA
TCCCTGATCAATGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAACGGATT
CTGCCCGCACAAGGTCTCCGCTGGGCAGTTTCCAGCTTGCGTGTCCGAGACACCAA

Exons: 1-112, 113-167, 168-272, 273-304

AGM IL-15, partial cds

CTTAAGGATTTACCGTGGCTTTGAGTAATGAGAATTTCGAAACCACATTTGAGAAGTGTTTC
CATCCAGTGCTACTTGTGTTTACTTCTAAACAGTCATTTTCTAACTGAAGCTGGCATTCATG
TCTTCATTTTGGGCTGTTTCAGTGCAGGGCTCCCTAAAACAGAAGCCAACTGGGTGAATGTA
ATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATGCATATTGATGCTACTTTATA
TACAGAAAGTGATGTTCACCCCAGTTGCAAGGTAACAGCAATGAAGTGCTTTCTCTTGGAGT
TGCAAGTTATTTCACATGAGTCCGGAGATACAGATATTCATGATACAGTAGAAAATCTCATC
ATCCTAGCAAACAACATCTTGTCTTCTAATGGGAATATAACAGAATCTGGATGCAAAGAATG
TGAGGAACTAGAGGAAAAAAATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAA
TGTTCATCAACACTTCTTGATTGCAACTGATTCTTTATAAAGTGTTTCTGTTACTAACGAAC
ATCACTCTGCTGCTTAGA

Exons: 1-38, 39-136, 137-222, 223-267, 268-404, 405-576IL-18

AGM IL-2, partial cds:

AGTAACCTCAACTCCTGCCACAATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTC
TTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTG
GAGCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAA
ACTCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAATTGAAACATC
TTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGCAAA
AACTTTCACTTAAGAGATACCAAGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACT
AAAGGGATCTGAAACAACACTGATGTGTGAATATGCTGATGAGACAGCAACCA

Exons: 1-169, 170-227, 228-374, 375-425

AGM IL-22, partial cds:

TGTGAGCCCTTTCCTTATGGGGACTCTGGCCACCAGCTGCCTCCTTCTCTTGGCCCTCTGGG
TGCAGGGAGGAGCAGCTGCGCCCATCAGCTCCCACTGCAGGCTTGACAAGTCCAACTTCCAG
CAGCCCTATATCACCAACCGCACCTTCATGCTGGCTAAGGAGGCTAGCTTGGCTGATAACAA
CACAGACGTTCGTCTCATTGGGGAAAAACTGTTCCGCGGAGTCAGTATGAGTGAGCGCTGCT
ATCTGATGAAGCAGGTGCTGAACTTCACCCTTGAAGAAGTGCTGCTCCCTCAATCGGATAGG
TTCCAGCCTTATATGCAGGAGGTGGTGCCCTTCCTGGCCAGGCTCAGCAACAGTCTAAGCAC
ATGTCACATTGAAGGTGATGACCTGCATATCCAGAAGAATGTGCAAAAGCTGAAGGACACAG
TGAAAAAGCTTGGAGAGAGTGGAGAGATCAAAGCAATTGGATCTGATT

Exons: 1-164, 165-232, 233-376, 377-440, 441-474

AGM IL-4, partial cds:

CTTTGTCCACGGACACAACTGCCATATCGCTTTACAGGAGATCATCGAAACTCTGAACAGCC
TCACAGAGCAGAAGACTCTGTGCACCAAGTTGACCGTAACGGACATCCTTGCTGCCTCCAAG
AACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCGACCGTGCTCCGGCAGTTCTACAGCCA
CCATGAGAAGGACACTCGCTGCCTGGGTGCAACTGCACAGCAGTTCCACAGGCACAAGAAGC
TGATCCGATTCCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGAGGGCTTGAGCTCCTGT
CCTGTGAAGGAAGCCAGCCAGAGTACGTTGGAAGACTTCTTGGAAAGGCTAAAGACGATCAT
GAGAGAGAAATATTCAAAGTGTTCGAGCTGAATA

Exons: 1-73, 74-122 (nur bei Var.1), 123-299, 300-406

AGM IL-5, partial cds:

TATGAGGATGCTTCTGCATTTGAGTTTGCTAGCTCTTGGAGCTGCTTACATGTATGCCATCC
CCACAGAAATTCCCACAAGTGCATTGGTGAAAGAGACCTTGGCACTGCTTTCTACTCATCGA
ACTCTGCTGTTAGGCAATGAGACTCTGAGGATTCCTGTTCCTGTACATAAACATCACCAACT
GTGCACTGAAGAAATCTTTCAGGGAATAGGCACACTGGAGAGTCAAACTGTGCAAGGGGGTA
CTGTGGAAAGACTATTCAAAAACTTGTCTTTAATAAAGAAATACATTGACGGCCGAAAAAAA
AAGTGTGGAGAAGAAAGACGGAGAGTAAACCAATTCCTAGACTACCTGCAAGAGATTC

Exons: 1-143, 144-178, 179-307, 308-368

AGM IL-6, partial cds:

AAGCCAGAGCTGTGCAGATGAGTACAAAAGTCCTGATCCAGTTCCTGCAGAAAAAGGCAAAG
AATCTAGATGCAATAACCACCCCTGAACCAACCACAAATGCCAGCCTGCTGACGAAGCTGCA
GGCGCAGAACCAGTGGCTGCAGGACA

Exons: 1-54, 55-150

AGM IL-7 variant 1, partial cds:

TTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTAGTTCTGTTGCCAGTAGCATCATCTG
ATTGTGATATTGAAGGACAGCATGAAAGAAATTGGTAGCAATTGCCTGAATAATGAATTTAA
CTTTTTTAAAAGACATCTATGTGATGATAATAAGTTTCAGAAGGCACAACAATACTTTTGAA
CTGCACCGGCAAGGTTAAAGGAAGAAAACCAGCTGCCCTGGGTGAACCCCAACCAACAAAGA
GTTTGGAAGAAAATAAATCTTTAAAGGAACAGAAAAAACTGAATGACTCATGTTTCCTAAAG
AGACTACTACAAAAGATAAAAACTTGTTGGAATAAAATTTTGATGGGCACTAAAGAACACTG
AAAAATATGGAGTGGCAATATAGAAACA

Exons: 1-75, 76-155, 156-196, 197-252, 253-400

AGM IL-7 variant 2, partial cds:

TTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTAGTTCTGTTGCCAGTAGCATCATCTG
ATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTTCTAATGGTCAGCATCGAT
CAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTGAATAATGAATTTAACTTTTT
TAAAAGACATCTATGTGATGATAATAAGGAAGGTATGTTTTTATTCCGTGCTGCTCGCAAGT
TGAAGCAATTTCTTAAAATGAATAGCACTGGTGATTTTGATCTCCACTTATTAAAAGTTTCA
GAAGGCACAACAATACTTTTGAACTGCACCGGCAAGGTTAAAGGAAGAAAACCAGCTGCCCT
GGGTGAACCCCAACCAACAAAGAGTTTGGAAGAAAATAAATCTTTAAAGGAACAGAAAAAAC
TGAATGACTCATGTTTCCTAAAGAGACTACTACAAAAGATAAAAACTTGTTGGAATAAAATT
TTGATGGGCACTAAAGAACACTGAAAAATATGGAGTGGCAATATAGAAACA

Exons: 1-132, 133-212, 213-343, 344-399, 400-547

AGM IL-8, partial cds:

CAAGAGCCAGGAAGAAACCACCGGAAGGAACCATCTCGCTGTGTGTAAACATGACTTCCAAG
CTGGCGGTGGCTCTCTTGGCAGCCTTCCTGCTTTCTGCAGCTCTGTGTGAAGGTGCAGTTTT
GCCAAGGAGTGCTAAAGAACTTAGATGTCAGTGCATAAAGACGTACTCCAAACCTATCCACC
CCAAATTTATCAAAGAACTGAGAGTGATTGAGAGTGGACCACACTGCGTCAATACAGAAATT
ATTGTAAAACTTTCCGATGGAAGAGAGCTCTGTCTGGACCCCAAGGTACCATGGGTGTCTAG
GGTTGTGGAGAAGTTTTTGAAGAGGGCTGAGAGTCAAAATTCATAAAAAAACAATGCATTCT
CCATGGTATCCAAGAATCAGTGAAGATGCCA

Exons: 1-112, 113-250, 251-335, 336~347, 348-403

AGM IP-10, partial cds:

CTACAGCAGAGGAATCTCCAGTCTCAGCACCATGAATCAAACTACCATTCTGATTTGCTGCC
TTGTCTTTCTGACTCTAAGTGGCATTCAAGGAATACCTCTCTCAAGAACTGTACGCTGTACC
TGCATCAGCATTAGTAATCAACCTGTTAATCCAAGGTCTTTAGAAAAACTTGAAATTATTCC
TCCAAGTCAATTTTGTCCACATGTTGAGATCATTGCTACAATGAAAAAGAAGGGTGAGAAGA
GGTGTCTGAATCCAGAATCTAAGGCCATCAAGAATTTACTGAAAGCAGTTAGCAAGGAAAGG
CCTAAAAGATCTCCCTAAAACCAGACGGAAACAAAGTGCTGCCAAGGATGGACCACACAGAG
GCTGCCTCTCCCATCACTTCCCTACATGGAGTATATGTCAAGCCCTAATTGTTCTTAGTTTG
CAGTTCCACTAAAAGGTGACCTATCATGGTCACCAACTCAGCTGCTCCTACTCCTGTAGGAA
GGTCAATGTTCATCATCCTAAGCTACTCAGTAATAACTCTACCCTGGTACTATAATGTAAGC
TGTACTGAGGTGCTATCTTCTTAGTGGATGTGCCAAGTCCTAACCCTGCTTCCAGTATTTCC
CTCACCTTTCCCATCTTCCAAGGGTACTAAGGAATCTTTCTGCTTTGGGGTTTATCAGAATT
CTCAGAATCTCAAATAACTAAAAGGCAATCAAAATGATAATACAATCTGCTTTTTAAAGAAT
GCCCTTACTTCATGGACTCCACTGCC

Exons: 1-90, 91-219, 220-307, 308-770

AGM IRF-7, partial cds:

GATCAGCAGCGGCTGCTATGAGGGGCTGCAGTGGCTGGACGAGGCCCGCACCTGCTTCCGCG
TGCCCTGGAAGCACTTCGCGCGCAAGGACCTGAGCGAGGCTGACGCGCGCATCTTCAAGGCC
TGGGCCGTGGCCCGCGGCAGGTGGCCGCCTAGCAGCAGAGGAGGTGACCCGCCGCCCCCCGA
GGCTGAGGCTGCGGAGCGCGCCAGCTGGAAAACCAACTTCCGCTGCGCACTGCGCAGCACGC
GCCGCTTCGTGATGCTGCGAGATAACTCGGGGGACCCGGCCGACCCGCACAAGGTGTACGCG
CTGAGCCCGGAGCTGGGCTGGCGAGAAGGCCCAGGAACGGACCAGACTGAGGCAGAGGCCCC
CGCGGCTGTCCGGGCACCGCAGGGCAGGCCCCCAGGGCCATTCCTGGCACACAGAGATGCTG
GACTCCAAGCCCCAGGCCCCCTCCCTGCCCCAGCTGGTGACAAGGGGGACCTCCTGCTCCAG
GCAGTGCAACAGAGCTGCCTGGCGGACCATCTGCTGACAGCGTCATGGGGGGCAGACCCAGT
CCCAGCCCAGGCTCCTGGAGAGGGACAAGAGGGTCTTCCCCTGACTGGGGCCTGTGCTGGAG
GTCCAGGGCTCCCTGCTGGGGAGCTGTGCACATGGGCAGTAGAAGCAACCCCTAGCCCCGGG
TCCCAGCCYGCAGCACTAATGACAGGCGAGGCCACGGCCCCAGAGCCCCTGCACCAGGTAGA
GCCATACCTGGCACCCTCCCCAAGTGCCTGCACTGCGGTGCAAGAGCCCAGCCCAGGGGCGC
TGGACGTGACCATCATGTACAAGGGCCGCACAGTGCTGCAGAAGGTGGTGGGGCACCCGAGC
TGCATGTTCCTGTACGGCCCCCCAGACCCAGCTGTCCGGGCCACAGACCCCCAGCAGGTAGC
ATTCCCCAGCCCTGCTGAGCTCCCCGACCAGAAGCAGCTGCGCTACACGGAGGAACTGCTGC
GGCACGTGGCCCCTGGGCTGCAGCTGGAGCTTCGGGGGCCACAGCTGTGGGCCCGGCGCATG
GGCAAGTGCAAGGTGTACTGGGAGGTGGGTGGCCCCCCGGGCTCCGCCAGCCCCTCCACCCC
AGCCTGCCTGCTGCCTCGGAACTGCGACACCCCCATCTTTGACTTCAGAGTCTTCTTCCGAG
AGCTGGTGGAATTCCGGGCACGGCAGCGCCGCGGCTCCCYATGCTATACCATCTACCTGGGC
TTCGGGCAGGACCTGTCAGCCAGGAGGCCCAAGGAGAAGAGCCTGGTCCTGGTGAAGCTGGA
GCCCTGGCTGTGCCGAGTGCACCTGGAGGGCACGCAACGTGAGGGTGTGTCTTCCCTGGATA
GCAGCAGCCTCAGCCTCTGTCTGTCCAGCACCAACAGCCTCTATGATGACATTGAGTGTTTG
CTCATGGAGCTGGAGCA

Exons: 1-119, 120-333, 334-390, 391-622, 623-704, 705-786, 787-1176, 1177-1295, 1296-1443

AGM MCP-1, partial cds

CGCCCTTTGTGCCTGCTGCTCATAGCAGCCACCTTCAGCCCGCAGGGGCTCGCTCAGCCAGA
TGCAATCAATGCCCCCGTCACTTGCTGCTATAACTTCACCAATAGGAAGATCTCAGTGCAGA
GGCTGGCGAGCTATAGAAGAATCACCAGCAGCAAGTGTCCCAAAGAAGCTGTGATCTTCAAG
ACCATTGTGGCCAAGGAGATCTGTGCTGACCCCAAGCAGAAGTGGGTTCAGGATTCCATGGA
CCACCTGGGCAAGCAAATCCAAACTCCGAAGCCTTGAA

Exons: 1-57, 58-179, 180-286

AGM MIP-1 alpha, partial cds:

GGCAACATTTGCTGCTGACACCCCGACCTCCTGCTGCTTCAGCTACATCTCCCGGCAGATTC
CACAGAATTTCGTAGCTGACTACTTTGAGACCAGCAGCCAGTGCTCCAAGCCCGGTGTCATC
TTCCTAACCAAGAGAGGCCGGCAGGTCTGTGCTGACCC

Exons: 1-4, 5-123, 124-162

AGM MIP-1 beta, partial cds:

CTGTGCGTGACTGTCCTGTCTCTCCTCGTGCTAGTAGCTGCCTTCTGCTCTCCAGCACTCTC
AGCACCAATGGGCTCAGACCCTCCCACCTCCTGCTGCTTTTCTTACACCGTGAGGAAGCTTC
CTCGCAACTTTGTGGTAGATTACTACGAGACCAGCAGCCTCTGCTCCCAGCCAGCTGTGGTA
TTCCAAACCAAAAGAGGGAAGCAAGTCTGCGCTGACCCCAGTGAGACCTGGGTCCAGGAGTA
TGTGTATGACCTGGAACTGAACTGAGCTGCTCAGAGACAGGAAGTCTTCAGGGAAGGTCACC
TGAGCCTGGATGCTTCTCCATGAGAGGCATCTCCTCCATACTCAGGACTCCTCTCCACAGTT
CCTGTCCCTTCTGTTAATTTAATCTTTTTAATGTGTCGTGTTATTGTATTAGGTGTTATTTC
CATTATTTATATTTGTTTAGCCAAAGTGTCTCCTATGGAGATGGTCCACCGTCA

Exons: 1-70, 71-185, 186-488

AGM PD1, partial cds:

AATCCCCGGACAGGCCCTGGAACCCCCCCACCTTCTCCCCAGCCCTGCTCCTGGTGACCGAA
GGAGACAACGCCACCTTCACCTGCAGCTTCTCCAACGCCTCGGAGAGCTTCGTGCTGAACTG
GTACCGCATGAGCCCCAGCAACCAGACAGACAAGCTGGCTGCCTTCCCCGAGGACCGCAGCC
AGCCCTGCCAGGACTGCCGCTTCCGCGTCACACAACTGCCCAATGGGCGCGACTTCCACATG
AGCGTGGTCAGGGCCCGGCGCAATGACAGCGGCACCTACCTCTGCGGGGCCATCTCCCTGGC
CCCCAAGGCGCAGATCAAAGAGAGCCTGCGGGCAGAGCTCAGGGTGACAGAGAGAAGGGCAG
AAGTGCCCACAGCCCACCCCAGCCCCTCACCCAGGCCAGCTGGCCAGTTCCAAGCCCTGGTG
GTCGGTGTCGTGGGCGGCCTGCTGGGTAGCCTGGTGCTGCTAGTCTGGGTCCTGGCCGTCAT
CTGCTCCCGGGCTGCACAAGGGACCATAGAAGCCAGGCGCACCGGCCAGCCCCTGAAGGAGG
ACCCCTCAGCCGTGCCTGTGTTCTCTGTGGACTATGGGGAGCTGGATTTCCAGTGGCGAGAG
AAGACCCCGGAGCCCCCCGTGCCCTGTGTCCCTGAGCAGACGGAGTACGCCACCATTGTCTT
TCCTGGTGGGCTGGGCACCTCGTCCCTGGCCCGCAGGGGCTCAGCCGACGGCCCTCGGAGTC
CCCGGCCACTGAGGCCGGAGGATGGACACTGCTCTTGGCCCCTCTGACCGGCTT

Exons: 1-357, 358-513, 514-550, 551-798

AGM PDL1, partial cds

TGGCATTTGCTGAATGCATTTACTGTCACAGTTCCCAAGGACCTATATGTGGTAGAGTATGG
CAGCAATATGACAATTGAATGCAAATTCCCAGTAGAAAAACAATTAGACCTGGCTTCACTAA
TTGTCTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTGCATGGAGAGGAAGACTTG
AAGGTTCAGCATACTAACTACAGACAGAGGGCCCAGCTGTTGAAGGACCAGCTCTCCCTGGG
AAATGCTGCACTTCAGATCACAGATGTGAAATTGCAGGATGCAGGGGTTTACCGCTGCATGA
TCAGCTATGGTGGTGCCGACTACAAGCGGATTACTGTGAAAGTCAATGCTCCATACAACAAA
ATCAACCAGAGAATTTTGGTTGTGGATCCAGTCACCTCTGAACATGAACTAACATGTCAGGC
TGAGGGCTACCCCAAGGCTGAAGTCATTTGGACAAGCAGTGACCATCAAGTCCTGAGTGGTA
AGACCACCACCACCGATTCCAAGAGAGAGGAGAAGCTTTTCAATGTGACCAGCACACTGAGA
GTCAACACAACAGCTAATGAGATTTTCTACTGCATTTTTAGGAGATTAGATCCTGAGGAAAA
CCATACAGCTGAATTGGTCATCCCAGAACTACCTCTGGCACCTCCTCCAAATGAAAGGACTC
ACTTGGTAATTCTGGGAGCCATCTTTTTACTCCTTGGTGTAGCACTGACATTTATCTTCTAT
TTAAGAAAAGGGAGAATGATGGATATGAAAAAATGTGGCATTCGAGTTACAAACTCAAAGAA
GCAAAGTGATACACAATTGGAGGAGAC

Exons: 1-14, 15-357, 358-643, 644-751, 752-813, 814-833
AGM PDL2, partial cds:

TTTGGCCAGGATACTTCTGCCAGAGGATAACCTGTAACCTGGCATGTGAGCTCTACCTCATT
TGTTTCTGGAACCTTTAGGATGTGAGTGTTTATTTTCCTGTAAGAAGCTTTGACTTTCAGAG
TCAGGTACTTGTAGTCCCAGGCAACCCCATATATGATTATGCTTTGGTACTGTCCTTCGTCC
CTCACTTGGACTTGAGGTATGTGGAACAAGGCCTTCCCTAGGGACAGCTGCTCCTCCAGCAA
AGTGGCTCTTTCACGGTGTGGGGATGTATCATTTTCCACCTTTTGCAAACTGGCTGTTATTG
CTCCAAGGTTCACATGACTTCCAGTGTCAAAGTTGCATTCCAGGGTCACATTGCTGCCATGC
TCTATTATGTACAGTTCCTTAGGGACTGTCACTGTGAATAAAGCTGCTGTCTGGTGAAGCTG
CAGTTCCAGGCTCAACATTAGCAGGAGGAAGATCATGTTCTGTATTTGATATGAGGACTTGT
CGCAGCTCCACAGGTAAAGATGCAAAAGAAAACAACAAAAAAACAGTTGCTGTTTGCGTTAG
ATTTCCTTATAGTAGCCACCAAAATGAACAGCCAAAAGGAAAAACAAAACCATCAACATGCA
GTAGCTCCAA

Exons: 1-109, 110-415, 416-484, 485-630

AGM RANTES, partial cds:

CCCTCTGCGCTCCTGCATCTGCCTCCCCACATGCCTCAGACACCACACCCTGCTGCTTTGCC
TACATTGCCCGCCCACTGCCCCGTGCCCACATCAAGGAGTATTTCTACACCAGTGGCAAGTG
CTCCAACCCAGCAGTCGTCTTTGTCACCCGAAAGAATCGCCAAGTGTGTGCCAACCCA

Exons: 1-182

AGM TNF-alpha, partial cds:

GAGCTGGCCGAGGAGGCGCTCCCCAGGAAGACAGCGGGGCCCCAGGGCTCCAGGCGGTGCTG
GTTCCTCAGCCTCTTCTCCTTCCTGCTCGTGGCAGGCGCCACCACGCTCTTCTGTCTGCTGC
ACTTTGGAGCGATCGGCCCCCAGAGGGAAGAGTTCCCCAAGGACCCCTCTCTATTCAGCCCT
CTGGCCCAGGCAGTCAGATCATCTTCTCGAACCCCAAGTGACAAGCCTGTAGCCCATGTTGT
AGCAAACCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGCCGGGCCAATGCCCTCCTGG
CCAATGGCGTAGAGCTGACAGATAACCAGCTGGTGGTGCCATCAGAGGGCCTGTACCTCATC

TACTCCCAGGTCCTCTTCAAGGGCCAAGGCTGCCCCTCCAACCATGTGCTCCTCACCCACAC
CATCAGCCGCATCGCCGTCTCCTACCAGACCAAGGTCAACCTCCTCTCTGCCATCAAGAGCC
CCTGTCAGAGGGAGACTCCAGAGGGGGCTGAGGCCAAGCCCTGGTATGAGCCCATCTACCTA
GGAGGGGTCTTTCAGCTGGAGAAGGGTGATCGACTCAGCGCTGAGATCAATCTGCCCGACTA
TCTCGACTTTGCCGA

Exons: 1-154, 155-202, 203-248, 249-632

AGM VEGF, partial cds:

AGACCCTGGTGGACATCTTCCAGGAGTACCCTGATGAGATTGAGTACATCTTCAAGCCATCC
TGTGTGCCCCTGATGCGATGTGGGGGCTGCTGCAATGACGAGGGCCTGGAGTGTGTGCCCAC
TGAGGAGTCAAACATCACCATGCAGATTATGCGGATCAAAC

Exons: 1-165

Functions of biomarkers investigated

Marker	Full name	Relevance in HIV/SIV infection
FoxP3	Forkhead box P3	Mainly expressed on regulatory T-cells
GCSF	Granulocyte colony-stimulating factor	Stimulates survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils
GM-CSF	Granulocyte macrophage colony-stimulating factor	Stimulates stem cells to produce granulocytes and monocytes
IFN alpha	Interferon alpha	Proinflammatory, inhibition of HIV replication by APOBEC3G upregulation, inhibition of apoptosis in activated T-cells, induction of apoptosis in uninfected T-cells, recruitment of target cells, NK-cell proliferation
IFN beta	Interferon beta	Proinflammatory, probably bystander product of IRF-7 activation
IFN gamma	Interferon gamma	Th1-type cytokine, proinflammatory
IL-1 alpha	Interleukin 1 alpha	Proinflammatory cytokine, atherosclerosis, cardiovascular disease
IL-1 beta	Interleukin 1 beta	Proinflammatory cytokine, activation of monocytes and macrophages, fever induction
IL-10	Interleukin 10	Th2-type cytokine, B-cell survival
IL-12 alpha	Interleukin 12 alpha	T-cell and NK-cell stimulation, enhancement of T- and NK-cell cytotoxic activity
IL-12 beta	Interleukin 12 beta	T-cell and NK-cell stimulation, enhancement of T- and NK-cell cytotoxic activity
IL-13	Interleukin 13	Th2-type cytokine, B-cell activation
IL-15	Interleukin 15	NK-cell activation

Marker	Full name	Relevance in HIV/SIV infection
IL-16	Interleukin 16	Inhibition of HIV/SIV transcription, chemoattractant for CD4
IL-17	Interleukin 17	Induction of antimicrobial peptide secretion
IL-18	Interleukin 18	Proinflammatory cytokine, atherosclerosis, cariovascular disease, induction of IFN gamma production (in the presence of IL-12) in Th1- NK- and B-cells
IL-1Ra	Interleukin 1 receptor antagonist	Competitive inhibition of IL-1 alpha and IL-1 beta, corresponds to delayed immune response upon IL-1 production
IL-2	Interleukin 2	Proliferation and differentiation of cytotoxic T-cells and NK-cells, T-regulatory cell development, B-cell activation
IL-22	Interleukin 22	Induction of antimicrobial peptide secretion, re-epithelialisation, pro- and anti-inflammatory cytokine
IL-4	Interleukin 4	Th2-type cytokine, B-cell proliferation
IL-5	Interleukin 5	Th2-type cytokine, B-cell proliferation, activation of eosinophils
IL-6	Interleukin 6	Proinflammatory, proliferation and differentiation of T- and B-cells, astrocytes, hepatocytes Upregulation of HIV-1 production in monocytes.
IL-7	Interleukin 7	Regulation of peripheral T-cell homeostasis, negative correllation with CD4+ T-cell levels
IL-8 (CXCL8)	Interleukin 8	Chemokine attracting neutrophils, competitively inhibiting HIV by binding to CXCR2
IP-10 (CXCL10)	Interferon gamma-induced protein 10	Chemokine attracting and activating NK-cells
IRF-7	Interferon regulatory factor 7	Transcription factor for IFN alpha

Marker	Full name	Relevance in HIV/SIV infection
MCP-1 (CCL2)	Monocyte chemotactic protein 1	Chemokine, recruitment of monocytes and memory T-cells, Th2-polarisation
MIP-1 alpha	Macrophage inflammatory protein 1 alpha	Competitively inhibiting HIV by binding to CCR5, proinflammatory, leukocyte chemotaxis, inhibits the proliferation of hematopoietic stem cells
MIP-1 beta	Macrophage inflammatory protein 1 beta	Competitively inhibiting HIV by binding to CCR5, proinflammatory, leukocyte chemotaxis, inhibits the proliferation of hematopoietic stem cells
PD-1	Programmed death 1	T-cell anergy, suggested involvement in memory B-cell depletion
PDL-1	Programmed death ligand 1	T-cell anergy, suggested downregulatiuon of activated T- and B-cells
PDL-2	Programmed death ligand 2	T-cell anergy, suggested downregulation of macrophages
RANTES (CCL5)	Regulated on Activation Normal T Expressed and Secreted	Chemokine, recruitment of eosinophils, monocytes, and lymphocytes. Competitively inhibiting HIV by binding to CCR5 and CCR3, Th1-polarisation
sCD40L	Soluble CD40 ligand	Suggested involvement in T-cell dependent B-cell activation, association with increased risk for cardiovascular disease
TNF alpha	Tumour necrosis factor alpha	Th1-type cytokine, inhibition of pDC generation, HIV inhibition through upregulation of acute-phase proteins
VEGF	Vascular endothelial growth factor	Involved in cardiovascular inflammation

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Comparison of anamnestic immune responses primed by Gag DNA gene-gun immunization of the African green monkey natural hosts and rhesus macaque heterologous hosts of simian immunodeficiency virus
Christine S. Siegismund, Janna Seifried, Oliver Hohn, Cheick Coulibaly, Roland Plesker, Reinhard Kurth and Stephen Norley (Submitted to *Journal of Virology* 2011)

Plasma cytokine and chemokine dynamics are distinct between acutely SIV-infected rhesus macaques and African green monkeys.
Janna Seifried, Christine S. Siegismund, Cheick Coulibaly, Roland Plesker, Reinhard Kurth and Stephen Norley (in preparation)

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HIV/SIV research is a rapidly growing field and yet it doesn't grow fast enough. With all the progress being made in the field of HIV vaccines and microbicides, I find it important not to forget those more than 30 million people who are already HIV-infected. Finding out the mechanism how natural hosts for SIV avoid the development of AIDS could give rise to a life-saving treatment for them. This being said, I would like to dedicate this thesis to a fellow exchange student during my time in South Africa, who was infected with HIV at the young age of 16 because her boyfriend lied about his status. I sincerely hope she is well.