

Cairo University
Faculty of Veterinary Medicine
Virology Department



جامعة القاهرة
كلية الطب البيطري
قسم الفيروسات

Analysis of Granzyme B- Expressing B Cells in SIV- Infected Rhesus Macaques

Thesis presented by
Ahmad Hassan Kotb
(B.V.Sc., Faculty of Vet. Med., Cairo University, 2011)

For the degree of M.V.Sc
(Virology)

Under the Supervision of

***Prof. Dr. Mohamed Abd
El-Hameed Shalaby***

Professor of Virology and Immunology,
Virology Department,
Faculty of Veterinary Medicine,
Cairo University

***Prof. Dr. Ahmed Abd El-Ghani
El-Sanousi***

Professor of Virology,
Virology Department,
Faculty of Veterinary Medicine,
Cairo University

Prof. Dr. Momtaz Abd El-Hadi Afify Shaheen

Chief Researcher, Virology Department
Head of the Animal Health Research Institute,
Dokki, Giza

2017

Supervision Sheet

Analysis of Granzyme B- Expressing B Cells in SIV-Infected Rhesus Macaques

Thesis presented by

Ahmad Hassan Kotb

(B.V.Sc., Faculty of Vet. Med., Cairo University, 2011)

For the degree of M.V.Sc

(Virology)

SUPERVISION COMMITTEE:

Prof. Dr. Mohamed Abd El-Hameed Shalaby

Professor of Virology and Immunology, Virology Department, Faculty of Veterinary Medicine, Cairo University.

Prof. Dr. Ahmed Abd El-Ghani El-Sanousi

Professor of Virology, Virology Department, Faculty of Veterinary Medicine, Cairo University.

Prof. Dr. Momtaz Abd El-Hadi Afify Shaheen

Chief Researcher, Virology Department, Head of the Animal Health Research Institute, Dokki, Giza.

Cairo University
Faculty of Veterinary Medicine
Department of Virology

Name : Ahmed Hassan Kotb
Birth date : 30/09/1989
Place of Birth : Cairo
Nationality : Egyptian
Scientific degree : Master's Degree of Veterinary Science (MVSc)
Specification : Virology and Immunology
Thesis title : Analysis of Granzyme B- Expressing B Cells in SIV-Infected Rhesus Macaques

Supervisors:

Prof. Dr. Mohamed Abd El-Hameed Shalaby

Professor of Virology and Immunology, Virology Department, Faculty of Veterinary Medicine, Cairo University.

Prof. Dr. Ahmed Abd El-Ghani El-Sanousi

Professor of Virology, Virology Department, Faculty of Veterinary Medicine, Cairo University.

Prof. Dr. Momtaz Abd El-Hady Afify Shaheen

Chief Researcher, Virology Department, Head of the Animal Health Research Institute, Dokki, Giza.

Abstract:

HIV infection continues to be a major global health issue and is characterized by a profound dysregulation of various immune cells, including B cells. Recently, increased frequencies of regulatory, granzyme B-expressing B cells have been identified in HIV-infected patients compared to healthy subjects, but their function remains unclear. Due to limitations in studies with HIV-infected individuals, animal studies are needed. To date, the experimental infection of rhesus macaques (*Macaca mulatta*) with simian immunodeficiency virus (SIV) is the best animal model for HIV/AIDS research. The aim of this work was to analyse frequencies, phenotype and the possible function of granzyme B-expressing B cells in healthy and SIV-infected rhesus macaques. Then B cells were purified using magnetic cell separation and different stimulation protocols were applied to induce granzyme B expression in vitro. Finally Co-culture experiments of these in vitro induced granzyme B-expressing B cells with T cells performed. Furthermore, we aimed at analyzing these cells in so-called long-term survivors (LTS), which lack disease progression in the absence of antiretroviral therapy, but can suddenly lose this status and progress to AIDS. By using multicolor flow cytometry the phenotype and frequencies of granzyme B-expressing B cells have been assessed and correlated with other immunologic parameters. Similar to HIV patients, significantly higher frequencies of these cells have been found in the blood of chronically SIV-infected rhesus monkeys compared with uninfected healthy ones. These frequencies correlated with plasma viral load and inversely with absolute CD4 T-cell counts. When investigating GrB⁺ B cells in different compartments, levels were highest in blood, spleen and bone marrow, but considerably lower in lymph nodes and tonsils. Analysis of expression of various surface markers on this particular B-cell subset in SIV-infected macaques revealed differences between the phenotype in macaques and in humans. GrB⁺ B cells in SIV-infected rhesus macaques exhibit an elevated expression of CD5, CD10, CD25 and CD27, while expression of CD19, CD185 and HLA-DR is reduced. In contrast to human GrB⁺ B cells, a significantly increased expression of CD43 and CD86 has not been observed. B-cell receptor stimulation in combination with IL-21 of purified B cells from healthy animals led to the induction of GrB expression. Furthermore, initial functional analyses indicated a regulatory role on T-cell proliferation. Overall, this data pave the way for longitudinal analyses including studies on the functionality of GrB⁺ B cells in the nonhuman primate model for AIDS.

Keywords: SIV, Rhesus Macaques, Long Term-Survivors, GrB⁺ B cells, Flow Cytometry.

*To my Family
and to
Philip Hagmann...*

*Who entered my Life and let me see everything from a
better, more beautiful perspective.*

Acknowledgment

First of all I am so Thankful for everyone, who helped and supported me in each step in this successful work and let it to happen.

*I wish to express my profound gratitude and high appreciation where all the words of acknowledgement come in short of describing my great indebtedness of thanks to **Prof. Dr. Mohamed Shalaby**, Professor of Virology and Immunology, Virology department; Faculty of Veterinary Medicine, Cairo University, for his keen supervision, faithful guidance, advice, unfailing patience and encouragement throughout the progress of work,*

*Sincere thanks are due to **Prof. Dr. Ahmed El-Sanousi**, Professor of Virology, Virology department; Faculty of Veterinary Medicine, Cairo University, for his keen supervision, valuable guidance, help and encouragement during the performance of the work,*

*I owe deep thanks to **Prof. Dr. Momtaz Shaheen**, Professor of Virology, Head of the Animal Health Research Institute, Dokki, for his keen and great help in delivering this work, keen supervision and encouragement.*

*I would like to thank **Dr. Christiane Stahl-Hennig**, Head of the Infection Models department, German Primate Center (DPZ), Göttingen, Germany, for the award of the Masters Project, and the chance to work in the department of infection models as a master's student.*

*I especially would like to thank **Dr. Berit Neumann**, German Primate Center (DPZ), Göttingen, Germany, for her willingness to cooperate in making this project happen, also for the introduction into the field of flow cytometry and the essential support in the analysis of the data as well as for her friendship.*

*I am so thankful for **Dr. Antonina Klippert**, German Primate Center (DPZ), Göttingen, Germany, for her help to answer all my questions concerning different experiments, Animal data and for her essential support in using different data analysis and statistical programs, as well as for her friendship and her endless motivation.*

*I would also like to thank my colleagues **Li Lin Gan and Maria Daskalaki** German Primate Center (DPZ), Göttingen, Germany, for their friendship, as well as for their valuable help and assistance whenever was needed.*

*I would also like to thank all my Friends, especially **Eslam Ali and Ahmed Nour El-Din** for their valuable help and assistance whenever was needed.*

I would also like to thank all my professors, colleagues and workmen for their help and willing to assist and cooperate.

Contents

<u>Contents:</u>	<u>Page:</u>
List of Table	I
List of Figures	II
Introduction	1
Review of Literature	3
1. HIV and AIDS	3
2. SIV-infected rhesus macaque as a model for HIV infection in humans	6
3. The Immune system	8
3.1 T cells	8
3.2 B cells	9
3.3 Granzyme B– expressing (GrB⁺) B cells	11
Materials and Methods	15
1. Media and reagents	15
2. Animals	18
3. Sample collection	22
3.1 Anaesthetization	22
3.2 Collection of blood samples	22
3.3 Collection of bone marrow by iliac crest aspiration (BMca)	23
3.4 Euthanasia and autopsy	24
4. Isolation of mononuclear cells (MNCs)	25
4.1 Isolation of MNCs from peripheral blood and BMca	25
4.2 Isolation of MNCs from bone marrow from the femur (BMfem) and secondary lymphoid organs (palatine tonsil, axillary and mesenteric lymph nodes (LN))	25
4.3 Isolation of MNCs from spleen	26
5. Cell counting	26
6. Flow cytometric analysis	27
6.1 Used monoclonal antibodies for flow cytometric analysis	27
6.2 Preparation and staining of cells	28
6.2.1 Preparation of cells for assessing granzyme B and IL-21 expression	28
6.2.2 FACS staining with directly fluorochrome-coupled antibodies	28
6.2.3 FACS staining with indirectly fluorochrome-coupled antibodies	29
6.2.4 Intracellular cytokine FACS staining	29

6.3	Staining protocols	30
6.3.1	Analysis of purity of magnetically separated B and T cells	30
6.3.2	Analysis of granzyme B-expressing B cells	30
6.3.3	Analysis of CD4 ⁺ T cells	31
6.4	Determination of absolute CD4 T cell counts	32
6.5	Cytometer and cell analysis	32
7.	Magnetic activated cell sorting (MACS)	33
7.1	Magnetic labeling of CD20 ⁺ B cells	33
7.2	Magnetic labeling of CD4 ⁺ cells	34
7.3	Magnetic separation	34
7.4	Analysis of purity of magnetically separated cells	34
8.	In vitro induction of granzyme B expression in purified CD20⁺ B cells	35
9.	Functional analysis of granzyme B-expressing B cells	36
9.1	Plate-bound CD3/CD28 stimulation of CD4 ⁺ T cells	36
9.2	CFSE staining of purified CD4 ⁺ cells	36
9.3	Co-culture of B cells and CD4 ⁺ cells	37
10.	Determination of plasma SIV RNA levels	37
11.	Statistical analysis	37
Results	38	
1.	Flow cytometric characterization of granzyme B-expressing (GrB⁺) B cells in rhesus macaque	38
1.1	Identification of GrB ⁺ B cells in the periphery as well as primary and secondary lymphoid organs of rhesus macaques	39
1.1.1	Frequencies of GrB⁺ and CD107a⁺GrB⁺ B cells in PBMCs are significantly elevated in SIV-infected rhesus macaques	41
1.1.2	Frequencies of GrB⁺ and CD107a⁺GrB⁺ B cells in Primary and Secondary Lymphoid Organs are significantly increased in SIV-infected rhesus macaques	43
1.2	Phenotypic characterization of GrB⁺ B cells in naïve and SIVmac251-infected rhesus macaques reveals differences from reported human GrB⁺ B cells..	45
1.3	CD154⁺IL-21⁺CD4⁺ T cell dependent GrB⁺ B cells development in SIV-infected rhesus macaques reveals differences from reported in human HIV	51
1.3.1	Frequencies of IL-21⁺ and CD154⁺ (CD40L) CD4⁺ T cells in PBMCs are elevated in SIV-infected rhesus macaques	53
1.3.2	IL-21 and CD154 (CD40L) expression on CD4⁺ T cells in primary and secondary lymphoid Organs of SIV-infected rhesus macaques	56
1.4	The relationship between disease progression and frequencies of GrB⁺ B cells in SIV-infected rhesus macaques	59
1.4.1	Frequencies of GrB⁺ B cells correlate with SIV viral load and inversely with absolute CD4⁺ T cell count	59
1.4.2	Frequencies of GrB⁺ B cells do not correlate with frequencies of IL-21⁺ and CD40L⁺ CD4⁺ T cells	61

1.4.3	Frequencies of GrB ⁺ B cells do not correlate with increased frequencies of CD69 ⁺ CD4 ⁺ T cells	62
1.4.4	Frequencies of GrB ⁺ B cells correlate with Central memory CD4 ⁺ T cells and inversely with Effector memory CD4 ⁺ T cell	65
2.	Stimulation of purified CD20 ⁺ B cells isolated from naïve rhesus macaques with IL-21 and various B-cell-stimulatory agents results in the in vitro induction of granzyme B expression	69
3.	GrB ⁺ CD20 ⁺ B cells reduce autologous CD4 ⁺ T cell proliferation in SIV-infected rhesus macaques	72
Discussion		75
1.	Increased peripheral GrB ⁺ B cell frequencies in SIV-infected rhesus macaques compared to healthy controls correlate with markers prognostic for disease progression	75
2.	GrB ⁺ B cell are also detectable in primary and secondary lymphoid organs of SIV-infected rhesus macaques	77
3.	Phenotypic characterization of GrB ⁺ B cells in rhesus macaques	79
4.	Reduced CD40L expression of CD4 ⁺ T cells upon in vitro re-stimulation in SIV-infected rhesus macaques indicates similar mechanisms for GrB induction in B cells as reported for humans	81
5.	B cell stimulation in the presence of IL-21 leads to in vitro induction of granzyme B expression in B cells of healthy rhesus macaques	83
6.	Possible regulatory, suppressive function of GrB ⁺ B cells on CD4 ⁺ T cell proliferation	84
Summary		85
References		87
List of Abbreviation		99
الملخص العربي		102
المستخلص		103

List of tables

Table	Page
Table 1: List of Media and Reagents used in this study	15
Table 2: Overview of healthy animals used in this study	20
Table 3: Overview of SIV-infected animals used in this study	21
Table 4: Monoclonal antibodies used for flow cytometric analysis	27
Table 5: Staining protocols to test purity of CD20 ⁺ B cells and CD4 ⁺ T cells	30
Table 6: Staining protocols to analyze frequency and phenotype of granzyme B-expressing B cells	30
Table 7: Staining protocol to analyze <i>in vitro</i> induction of granzyme B-expressing B cells	31
Table 8: Staining protocol to analyze IL-21 ⁺ CD4 ⁺ T cells	31
Table 9: Staining protocol to analyze proliferation of CD4 ⁺ T cells	32
Table 10: Frequencies of naïve and memory T cell subsets in healthy and SIV-infected animals	67

List of figures

Figure	Page
Figure 1: Worldwide distribution of HIV infections.	4
Figure 2: The Classical three-phase course of HIV infection.	5
Figure 3: Viral kinetics of SIV compared to HIV.	7
Figure 4: B cell differentiation in the presence of full T cell help compared with incomplete T cell help during HIV infection.	13
Figure 5: Possible immunological functions of granzyme B-secreting B cells.	14
Figure 6: Representative gating strategy of GrB ⁺ and CD107a ⁺ GrB ⁺ B cells in PBMCs and MNCs from indicated organs in rhesus macaques.	40
Figure 7: Frequencies of GrB ⁺ and CD107a ⁺ GrB ⁺ B cells in PBMCs.	42
Figure 8: Frequencies of GrB ⁺ and CD107a ⁺ GrB ⁺ B cells in the periphery as well as primary and secondary lymphoid organs of SIV-infected rhesus macaques.	43
Figure 9: Phenotypic Characterization of GrB ⁺ CD20 ⁺ B cells.	48
Figure 10: Representative gating strategy shows that CD107a ⁺ GrB ⁺ B cells are not expressing IL-10.	49
Figure 11: GrB ⁺ CD20 ⁺ B cells also express higher frequencies of IgD.	50
Figure 12: Representative gating strategy of IL-21 ⁺ CD4 ⁺ T cell and CD154 ⁺ CD4 ⁺ T cells in PBMCs in rhesus macaques.	52
Figure 13: Frequencies of IL-21 ⁺ cells and CD154 ⁺ cells of CD4 ⁺ T cells in PBMCs.	55
Figure 14: Frequencies of IL-21 ⁺ cells and CD154 ⁺ cells of CD4 ⁺ T cells in the periphery as well as primary and secondary lymphoid organs.	58
Figure 15: Frequencies of GrB ⁺ B cells correlate with SIV plasma viral load and inversely correlate with absolute CD4 ⁺ T cells counts.	60
Figure 16: Frequencies of GrB ⁺ B cells do not correlate with the frequencies of IL-21 ⁺ CD154 ⁺ (CD40L ⁺) T cells.	61
Figure 17: Elevated frequencies of CD69 ⁺ cells of CD3 ⁺ , CD4 ⁺ and CD8 ⁺ T cells in PBMCs of SIV-infected rhesus macaques.	63
Figure 18: Frequencies of GrB ⁺ B cells do not correlate with the frequencies of CD69 ⁺ CD3 ⁺ , CD4 ⁺ T and CD8 ⁺ T cells.	64
Figure 19: Elevated frequencies of effector memory T cells in PBMCs of SIV-infected rhesus macaques.	66
Figure 20: Correlation between GrB ⁺ B cell frequencies and CD4 ⁺ T cells memory subsets.	68
Figure 21: Increased GrB expression in purified B cells upon stimulation with CpG or anti-BCR and IL-21.	71
Figure 22: Representative gating strategy of CFSE-stained CD4 ⁺ T cells in co-culture experiments with GrB ⁺ B cells.	73
Figure 23: Decrease of CFSE ^{low} CD4 ⁺ T cell frequencies upon co-culture with different ratios of GrB ⁺ B cells in rhesus macaques	74

Introduction

HIV/AIDS is considered to be one of the most devastating infectious diseases that have emerged in recent history (**Barré-Sinoussi, 1983**). Approximately, 36.9 million people are living with HIV, including 2 million people, who had been recently infected, and 1.2 million people having died from AIDS-related causes in 2014 (**WHO**).

HIV infection results in a significant dysregulation of $CD4^+$ and $CD8^+$ T cells, dendritic cells and B cells (**Pantaleo, 1996**). Dysregulation of B cells leads to high numbers of polyclonal activated B cells with hyperreactivity and hypergammaglobulinemia (**Moir, 2009**). These hyperactive B cells are characterized by impairment in neoantigen and recall antigen B cell responsiveness (**Moir, 2009; Shirai, 1992**).

Recently, a rare subset of B cells, called B regulatory cells (Bregs), was identified in mice and humans (**Blair; Mizoguchi, 2002**). These Bregs were demonstrated to be Interleukin (IL-10) and granzyme B (GrB) producers (**Vadasz, 2014**), although the latter commonly represents a major key component of NK (natural killer) cells and CTLs (cytotoxic T lymphocytes) (**M. Hagn, and Jahrsdörfer, B., 2012**).

So far, the immunological function of these granzyme B-expressing (GrB^+) B cells remains elusive and may range from antiviral or cytotoxic and autoregulatory to regulatory functions (**Kaltenmeier, 2015; Vadasz, 2014**). Recent studies discovered a large number of circulating GrB^+ B cells in the peripheral blood of HIV patients, although they are negligible in healthy people (**Kaltenmeier, 2015**).

As studies with HIV-infected individuals are limited regarding routine sampling or collecting samples other than blood, animal studies are needed. So far, the most widely used animal model for HIV research is the experimental infection of rhesus macaques (*Macaca mulatta*) with Simian immunodeficiency virus (SIV).

This thesis shows for the first time the existence of GrB⁺ B cells in rhesus macaques and aimed at analyzing these cells in both naïve and SIV-infected rhesus macaques. Using multicolor flow cytometry frequencies of GrB⁺ B cell in the periphery as well as primary and secondary lymphoid organs were assessed and a comprehensive phenotypic characterization was performed. Furthermore, possible correlations with markers prognostic for disease progression and studies regarding their functionality were performed.

Review of Literature

1. HIV and AIDS

In 1981, the United States of America announced more frequent reports about a newly acquired immune deficiency syndrome (AIDS) among homosexual men and drug addicts, which is clinically manifested by mucosal candidiasis, *Pneumocystis carinii*-pneumonia (PCP) (current nomenclature: *Pneumocystis jiroveci*), multiple viral infections and the development of Kaposi's sarcoma (**Gottlieb, 1981; Masur, 1981; Siegal, 1981**). During this epidemic, USA health authorities suggested that AIDS is accompanied with lowering the lymphocyte count, especially CD4⁺ T helper cells.

In the following years, weekly reports of the Center for Disease Control and Prevention (CDC) revealed new cases of AIDS (**CDC, 1981**) until they discovered the causative virus for this disease in 1983. In 1986 this T-lymphotropic virus was finally designed as Human Immunodeficiency Virus (HIV) (**Coffin, 1986**).

In 1986 a new HIV was isolated from a patient from Senegal (**Clavel, 1986**) and named HIV-2. HIV was then renamed analogously HIV-1.

So far, the origin of HIV-1 and HIV-2 remains elusive. The most common and main theory is different simian immunodeficiency virus (SIV) strains represent precursors for both types of HIV, which have been transferred from monkeys to humans as a zoonotic disease, and have been adapted to suit the human host. It is unclear how exactly these events have taken place. Causes might have been the consumption of monkey meat especially in Africa.

According to the most recent reports in 2015, 37 million are now living with HIV (WHO/UNAIDS). Sub-Saharan Africa remains the most affected region, with nearly 1 in every 20 adult living with HIV and accounting for nearly 71% of the people living with HIV worldwide (WHO 2013) (Fig. 1).

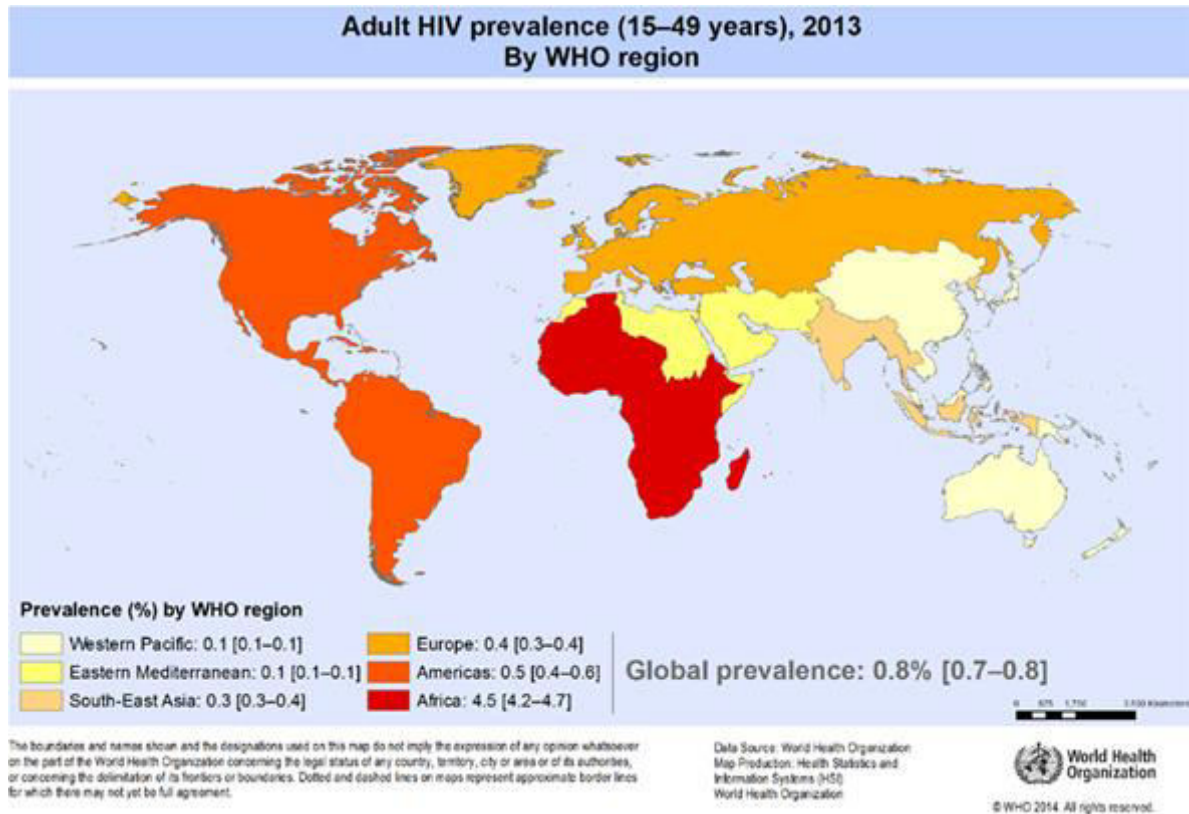


Figure 1: Worldwide distribution of HIV infections. According to UNAIDS and WHO AIDS epidemic update 2013.

Untreated HIV infections undergo a classical three-phase course of infection (Fig. 2) (Pantaleo, 1996). In most cases, the acute phase of the disease starts few days to few weeks after infection with flu-like symptoms such as fever, malaise, lymphadenopathy, weight loss and myalgia and lasts up to four weeks (Cooper, 1985). This phase is mainly characterized by a general immune activation accompanied with high viral load reaching peak viremia. In addition, there is a significant massive loss of CD4⁺ T cells, especially in the intestinal mucosa, which was first described in the SIV model in rhesus macaques (*Macaca mulatta*, RM) (Kewenig, 1999; Veazey RS, 1998) and later was confirmed in HIV patients (Brenchley, 2004; Guadalupe, 2003; Lim, 1993).