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HIV-1 GP120 MEDIATED MODULATION OF CD4$^+$ T CELL FUNCTION DURING HIV-1 INFECTION

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Summary

ENGLISH SUMMARY

Progressive decline of $CD4^+$ T cell function during HIV-1 infection is associated with a slow but steady deterioration of the immune system which ultimately leads to AIDS. Mechanisms explaining $CD4^+$ T cell dysfunction are manifold and still incompletely understood. One possible factor is attributed to HIV's ability to directly interfere with the function of $CD4^+$ T cells, based on the high affinity interaction between HIV-1's envelope glycoprotein gp120 and the CD4 receptor.

The results of this thesis demonstrate that a selective binding of gp120 or gp120-anti-gp120 immune complexes (ICs), which were documented to be abundantly present in HIV-1 patients, to the CD4 receptor can either enhance or diminish T cell receptor (TCR) signaling efficiency. Furthermore, we demonstrate that these opposing effects depend on the relative orientation of CD4 and TCR engagement, whereas synchronous and spatially linked CD4/TCR cross-linking by plate immobilized gp120-anti-gp120 ICs/anti-human CD3 mAbs promoted full $CD4^+$ T cell activation by exceeding local thresholds for signaling propagation, non-focal gp120-anti-gp120 IC binding to CD4 receptors undermines their capacity in supporting proximal signaling beyond the engaged TCRs and hence $CD4^+$ T cell activation. Mechanistically, we show that $CD4^+$ T cells efficiently capture gp120-anti-gp120 ICs from antigen-presenting cells (APC) and that such ICs bound to CD4 T cells interfere with the formation of immunological synapses (IS), representing a crucial step in TCR-induced $CD4^+$ T cell activation. Moreover, we show that $CD4^+$ T cells from HIV-1 patients are covered with ICs which contain gp120.

All in all, the data presented in this thesis suggest that binding of gp120-anti-gp120 ICs to CD4 receptors contribute to the progressing decline of $CD4^+$ T cell function during HIV-1 infection and thereby suggest a way of how HIV-1 destroys the immune system by its interference with $CD4^+$ T cell function at the level of TCR signaling.

GERMAN SUMMARY

Die HIV-1-Infektion ist durch eine fortschreitende Abnahme der $CD4^+$ T-Zellfunktion charakterisiert, die eine langsame aber stetige Verschlechterung des Immunsystems mit sich bringt und schlussendlich zu AIDS führt. Mechanismen, die einen solchen kontinuierlichen Rückgang der $CD4^+$ T-Zellen verursachen, sind vielfältig und bis heute noch nicht vollständig geklärt. Die Fähigkeit von HIV direkt mit der Funktion der $CD4^+$ T-Zellen zu interferieren, beruht möglicherweise unter anderem auf der hohen Affinität, die das Glykohüllprotein gp120 von HIV-1 für den CD4 Rezeptor hat.

Die Ergebnisse der Studien dieser Dissertation zeigen, dass eine selektive Bindung von gp120-Immunkomplexen, die bei HIV-1- Patienten reichlich vorhanden sind, an den CD4-Rezeptor die Signalübermittlung durch den T-Zell-Rezeptor schwächen oder verstärken kann. Darüber hinaus zeigen wir, dass diese gegensätzlichen Effekte von der relativen Ausrichtung der CD4 und T-Zell-Rezeptor-Stimulierung abhängen. Wenn nämlich die Stimulierung der CD4- und T-Zell-Rezeptoren an einer definierten Stelle erfolgt, indem gp120-Immunkomplexe und anti-CD3-Antikörper auf einer Platte immobilisiert werden, können die lokalen Schwellenwerte für die Signalweiterleitung überschritten werden und zur kompletten Aktivierung der $CD4^+$ T-Zellen führen. Im Gegensatz dazu verhindern gp120-Immunkomplexe, die ungerichtet an CD4-Rezeptoren binden, die die Signalübertragung durch den T-Zell-Rezeptor zu unterstützen. Wir zeigen mechanistisch, dass gp120-Immunkomplexe von Antigen-präsentierenden Zellen effizient auf $CD4^+$ T Zellen übertragen werden und dann mit der Ausbildung der immunologischen Synapse interferieren, einem essentiellen Schritt in der T Zell Rezeptor-vermittelten T Zell Aktivierung.

Zudem zeigen wir, dass $CD4^+$ T- Zellen von HIV-1-Patienten gp120-Immunkomplexe auf ihrer Oberfläche haben und somit zur Beeinträchtigung der $CD4^+$ T-Zellfunktion beitragen könnten.

Zusammenfassend zeigen die Resultate dieser Dissertation, dass die Bindung von gp120-Immunkomplexen an CD4-Rezeptoren die fortschreitende Abnahme der $CD4^+$ T-Zellfunktion während

der HIV -1-Infektion verursachen könnte über direkte Beeinträchtigung auf der Ebene der T-Zell-Rezeptor-Signalwege.

General Introduction

THE HIV-1 PANDEMIC

THE DISCOVERY OF HIV IN 1981

In 1981, the Center for Disease Control denoted the sudden occurrence of rare opportunistic infections such as Pneumocystis carinii pneumonia, mucosal candidiasis and Kaposi's sarcoma among a group of homosexual men in epidemic proportions (Friedman-Kien 1981, Gottlieb 1998). Since these men were not known to have any compromised immune system explaining the incidence of these diseases, intense research on that astonishing acquisition of immunodeficiency was initiated. Only one year later, the Center for Disease Control named this new disease "Acquired immune deficiency syndrome" (AIDS), according to the fact that the immune system seemed to become unable to protect from harmful or commensal microorganisms as well as transformed cells. In 1983, the human immunodeficiency virus (HIV) was discovered (Barre-Sinoussi, Chermann et al. 1983) and shortly thereafter clearly identified as the causative agent of AIDS (Gallo, Salahuddin et al. 1984, Levy, Hoffman et al. 1984). This virus spread predominantly in Central and East Africa as well as in Europe and the US. A related virus was discovered slightly later in patients originating from West Africa (Clavel, Guetard et al. 1986) suffering from an alleviated form of AIDS (Leligdowicz and Rowland-Jones 2008) and accordingly these two HIVs were denominated as HIV-1 and HIV-2, respectively.

THE ORIGIN OF THE HIV PANDEMIC

HIV-1 and HIV-2 arose as a consequence of multiple cross-species zoonotic transmissions of simian immunodeficiency viruses (SIVs) from naturally infected African primates to humans (Hahn, Shaw et al. 2000, Sharp 2002). SIV_{cpz} and SIV_{gor} which are phylogenetically close to HIV-1 were identified in some sub-species of non-natural hosts of SIV, namely in chimpanzee (Gao, Bailes et al. 1999, Santiago, Rodenburg et al. 2002, Keele, Van Heuverswyn et al. 2006) and lowland gorillas (Plantier, Leoz et al. 2009) which suffer from an AIDS-like disease (Paiardini and Muller-Trutwin 2013). In contrast, SIV_{sm} and SIV_{agm} were found to be circulating in sooty mangabey monkeys and African green monkeys (Santiago, Range et al. 2005) which don't progress to a disease (Silvestri, Paiardini et al. 2007). Consequently, it seems that the relatively recent introduction of HIV into humans or SIV into non-natural hosts causes an AIDS like disease, probably

reflecting a disruption of the stable and well adapted equilibrium between SIV and its natural hosts (Lisco et al, 2009).

HIV-1 PANDEMIC TODAY AND FUTURE GOALS OF HIV RESEARCH

Despite more than 30 years of extensive research on HIV, AIDS is undeniably one of the most devastating infectious diseases which is affecting human mankind (Barre-Sinoussi, Ross et al. 2013). HIV-1 established a fatal pandemic which to date caused 75 million infections [63 million–89 million] and 36 million [30 million – 42 million] AIDS-related deaths (UNAIDS Global Report 2013). Yet, the current knowledge of HIV pathogenesis allowed the development of potent combination antiretroviral therapy (ART) which offers great improvement of health but does not fully restore immune health. Especially inflammation-associated complications such as fibrosis in lymphoid tissues affecting the heart, lung, liver and kidney cause cardiovascular disease and cancer (Deeks, Lewin et al. 2013). Therefore, an urgent goal of HIV research is the development of an HIV-1 vaccine based on broad and potent HIV-1 neutralizing antibodies (McCoy and Weiss 2013; (Klein, Mouquet et al. 2013). Recent vaccine trials in non-human primates (Hessell, Rakasz et al. 2009, Watkins, Siddappa et al. 2011) raised hope in the field, since neutralizing antibodies were shown to be protective against new infections. However, the incomplete understanding of the viral-host interaction might still pose an obstacle in current vaccine trails and other therapeutic interventions of disease progression and needs to be further understood (Montagnier 2010).

HIV-1 STRUCTURE AND LIFE CYCLE

VIRAL STRUCTURE

HIV is an enveloped, spherical RNA virus which belongs to the genus of Lentivirus in the family Retroviridae according to their slow, progressive type of infection (Gonda 1988). The HIV genome consists of two identical 9181 bp long single-stranded positive sense RNA molecules which encode the typical retrovirus proteins Gag, Pol and Env (Gonda 1988). The *ENV* gene codes for the glycoprotein precursor gp160 which is processed by a host cell protease to form the surface envelope glycoprotein gp120 and transmembrane envelope glycoprotein gp41 (Stein and Engleman 1990). Post-translational modifications, such as disulfide bonds and N-linked glycosylation greatly influence folding, processing, and intracellular transport (Otteken, Earl et al. 1996, Zhang, Gaschen et al. 2004, Stansell and Desrosiers 2010), ultimately allowing the formation of a trimeric envelope structure consisting of non-covalently attached gp120 and gp41 molecules (Mao, Wang et al. 2012). *GAG* is translated into a polypeptide which is subsequently processed into matrix (p17) and capsid (p24) proteins. P17 and p24 form the viral core surrounding the viral genomic material as well as the viral enzymes which are encoded by *POL* an yield the protease, the error-prone reverse transcriptase (Preston, Poiesz et al. 1988) and the integrase (Frankel and Young 1998). Furthermore, HIV-1 possesses at least six regulatory genes, which provide regulatory gene function (tat and rev), assist in the assembly of the virion (vpu) and increase HIV's pathogenicity (vif, vpr and nef) (Frankel and Young 1998).

TROPISM

The CD4 receptor is the principal determinant of HIV-1 tropism (Dalgleish, Beverley et al. 1984, Sattentau 1988), however only the sequential interaction with an additional chemokine co-receptor CC chemokine receptor 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) trigger the fusion of viral and cellular membranes (Wells, Proudfoot et al. 1996, Berger, Murphy et al. 1999, Lee, Sharron et al. 1999). CCR5 allows the infection of primary $CD4^+$ T cells and macrophages (Dragic, Litwin et al. 1996) and represents the predominant co-receptor exploited for transmission and replication *in vivo.* In some HIV-1 variants, a switch to CXCR4 usage (X4 HIV-1 strains) occurs which is associated with faster disease progression (Feng, Broder et al. 1996). Prior interactions with the integrin $\alpha 4 \beta 7$ (Arthos, Cicala et al. 2008) or other adhesion

molecules might capture and consequently enhance viral attachment to the cell and thereby facilitate the viral entry process and influence the viral tropism (Bastiani, Laal et al. 1997, Cicala, Arthos et al. 2011). For example, gp120 binding to $\alpha 4\beta 7$ expressed on gut intestinal $CD4^+$ T cells (Arthos, Cicala et al. 2008) causes their fast and selective depletion early after HIV-1 infection (Guadalupe, Reay et al. 2003, Brenchley, Schacker et al. 2004, Mehandru, Poles et al. 2004, Gordon, Cervasi et al. 2010).

HIV-1 LIFE CYCLE

The surface part of the trimeric envelope glycoprotein gp120 binds with high affinity to the CD4 receptor (Myszka, Sweet et al. 2000) which represents the first step of HIV-1's life cycle. The high affinity gp120-CD4 receptor interaction causes crucial conformational changes in the envelope (Sattentau and Moore 1991, Wyatt and Sodroski 1998) which accordingly allow its interaction with a chemokine receptor CCR5 or CXCR4 (Berger, Murphy et al. 1999, Lee, Sharron et al. 1999). This in turn induces the insertion of the N-terminal hydrophobic end of the gp41 - the fusion peptides - into the host cell membrane, allowing the entry of the virus nucleocapsid. Once inside the host cell, a reverse transcription complex is formed to enable the synthesis of double stranded DNA which, with the help of the integrase, is inserted into the host genome. The so called provirus serves as template for the transcription mediated by the host RNA polymerase II. Two regulatory proteins Rev and Tat control HIV-1 gene expression, whereas Tat stimulates the transcription elongation, Rev promotes the transport of unspliced and incompletely spliced mRNA from the nucleus to the cytoplasm (Karn and Stoltzfus 2012). RNA molecules either serve as new viral genetic material or are translated into structural proteins and enzymes necessary for the formation of new viral particles. For viral propagation, viral matrix and core (p17 and p24) proteins, two linear viral RNA molecules, viral enzymes and envelope spikes are targeted to the site of virion assembly from where mature HIV particles are released by budding (Wilen, Tilton et al. 2012).

HIV-1 PATHOGENESIS

HIV-1 infection is associated with a progressive disturbance of immune system function which leads to increased susceptibility to opportunistic infections and an impaired ability of the immune system to suppress the reactivation of persistent microbes (Lisco, Vanpouille et al. 2009, Virgin, Wherry et al. 2009, Ng, Snell et al. 2013), ultimately causing a fatal state of immunodeficiency.

DISEASE PROGRESSION RATES

Although the disease progression of untreated HIV-1 infection is very dynamic and variable, most individuals eventually progress to AIDS. Whereas so called fast progressors develop AIDS in less than 3 years, long-term non progressors (LTNP) maintain high $CD4^+$ T cell numbers what is associated with a favorable outcome of the disease. A small fraction of these LTNPs are named "elite controllers" who are able to control plasma viremia to undetectable levels for prolonged time periods (Kumar 2013). The basis for such diverse progression rates is still incompletely understood, but might most likely include viral, genetic or immunological components. Non-progression can be partially attributed to non-functional CCR5 receptors (CCR5-Δ32) which are required for viral entry of R5 HIV-1 strains and consequently don't allow infection of $CD4^+CCR5^+$ T cells which are HIV's major target cells (Quillent, Oberlin et al. 1998).

ACUTE PHASE - HIV-1 REPLICATION IS CONTROLLED BY THE IMMUNE SYSTEM

Upon transmission, HIV-1 starts to replicate locally in the vaginal or rectal mucosa within resident memory $CD4^+CCR5^+$ T cells (Paiardini, Frank et al. 2008, Salazar-Gonzalez, Salazar et al. 2009). Of note, the specific affinity of gp120 for the gut homing receptor $\alpha 4\beta 7$ allows HIV-1 to selectively infect $CD4^+CCR5^+$ T cells resident in the gut intestinal mucosa (Arthos, Cicala et al. 2008, Kader, Wang et al. 2009, Ansari, Reimann et al. 2011) whose depletion is selective and very pronounced during the primary phase of HIV-1 infection (Guadalupe, Reay et al. 2003, Brenchley, Schacker et al. 2004, Mehandru, Poles et al. 2004, Gordon, Cervasi et al. 2010). Viral dissemination is highly facilitated by dendritic cells (DC) which capture HIV-1 through their binding to the c-type lectin DC-SIGN in the peripheral mucosa and subsequently transport them to susceptible target cells residing in lymphoid tissues(Geijtenbeek, Kwon et al. 2000). Plasma viremia

increases exponentially to reach a peak of more than a million RNA molecules per ml of blood usually around 3 to 4 weeks after infection (McMichael, Borrow et al. 2010).

Such massive HIV-1 replication provides stimuli that trigger innate immunity. Namely, HIV-1 ssRNA is recognized by TLR7/8 on innate immune cells (e.g. DCs, NK cells) which induces the production of antiviral and immunomodulatory cytokines (Diebold, Kaisho et al. 2004). Most prominent is the production of IFN-α by plasmacytoid DC (pDC), however a direct anti-viral activity could not be demonstrated so far (Kader, Smith et al. 2013). Stimulation of innate immune cells also leads to the production of IL-15, IL-18, IL-22, CXCL-10, TNF-α and IFN-γ (Gaines, von Sydow et al. 1990, Graziosi, Gantt et al. 1996, Stacey, Norris et al. 2009) with IL-15 inducing the activation and expansion of NK cells (Mueller, Do et al. 2008). NK cells contribute to viral control by direct cytolysis or indirectly through the production of antiviral cytokines and chemokines (Alter, Teigen et al. 2007); nevertheless, direct evidence demonstrating reduction of viral replication is still missing.

The efficient induction of HIV-1-specific cytotoxic T lymphocyte (CTL) responses (Borrow, Lewicki et al. 1994, Koup, Safrit et al. 1994) causes a decrease of HIV-1 replication to a viral set-point (McMichael, Borrow et al. 2010) as well as a transient recovery of $CD4^+$ T cell numbers in the perihphery. Recently, direct cytotoxic potential was also attributed to $CD4^+$ T cells (Soghoian, Jessen et al. 2012). Despite such a strong HIV-directed immune response, HIV-1 is not completely eradicated and establishes a life-long persistent infection in resting memory $CD4^+$ T cells which upon reactivation would promote active viral replication (Chun, Stuyver et al. 1997, Finzi, Hermankova et al. 1997, Finzi, Blankson et al. 1999, Blankson, Persaud et al. 2002, Eisele and Siliciano 2012). The HIV-specific B-cell response and the production of neutralizing antibodies does not contribute to the confinement of initial viral replication due to their delayed development (Frost, Trkola et al. 2008, McMichael, Borrow et al. 2010).

CHRONIC PHASE - CONTINUOUS HIV-1 REPLICATION IS ASSOCIATED WITH PROGRESSIVE IMMUNODEFICIENCY

Although the initial CTL-mediated HIV-specific immune response is highly efficient in confining viral replication, there is evasion from the cellular immune response during the chronic phase of the infection (Collins and Baltimore 1999). This is predominantly caused by mutations in the CTL epitopes (Preston,

Poiesz et al. 1988, Bernardin, Kong et al. 2005) as well as the down-regulation of MHC I molecule (Schwartz, Marechal et al. 1996, Collins, Chen et al. 1998, Yang, Nguyen et al. 2002). Similar as in the acute phase of the infection, neutralizing antibodies provide no substantial benefit in confining viral replication (Doria-Rose 2010) and on top of that there is a strong selection of HIV-1 variants in response to pressure from CTLs and neutralizing antibodies (Kearney, Maldarelli et al. 2009, Henn, Boutwell et al. 2012).

Constant decline of $CD4^+$ T cell numbers (Hazenberg, Hamann et al. 2000, McCune 2001, Grossman, Meier-Schellersheim et al. 2002, Okoye and Picker 2013) and a systemic activation of the immune system (Douek et al, 2009; Klatt et al, 2013; Paiardini & Muller-Trutwin, 2013) contribute to the progressive immunodeficiency which affects T cell (Clerici, Stocks et al. 1989, Dolan, Clerici et al. 1995, Brenchley, Karandikar et al. 2003, El-Far, Halwani et al. 2008) as well as B cell function (Malaspina, Moir et al. 2005, Moir and Fauci 2009). Importantly, the level of immune activation, reflected by high expression of activation markers on T cells (Giorgi, Liu et al. 1993) and B cells (Lane, Masur et al. 1983), increased T cell turnover (Hellerstein, Hanley et al. 1999, Silvestri and Feinberg 2003), polyclonal B cell expansion (Moir and Fauci 2013), elevated serum levels of pro-inflammatory cytokines and chemokines (Valdez and Lederman 1997, Biancotto, Grivel et al. 2007) as well as inflammatory damage of lymphoid tissues (Estes 2013), is considered as best marker for disease progression (Giorgi, Hultin et al. 1999, Hazenberg, Otto et al. 2003, Sachdeva, Fischl et al. 2010). Microbial translocation is one of multiple factors that are suggested to contribute to the chronic activation of the immune system (Brenchley, Price et al. 2006). The selective depletion of gut intestinal $CD4^+$ T cells which is very pronounced in the primary phase of HIV-1 infection (Guadalupe, Reay et al. 2003, Brenchley, Schacker et al. 2004, Mehandru, Poles et al. 2004, Gordon, Cervasi et al. 2010) is maintained during the chronic stage of the disease and provokes a loss of gut intestinal epithelial integrity (Bettelli, Korn et al. 2007). Such a loss of gut barrier function allows the transfer of gut luminal microbial bacteria to the systemic circulation (Brenchley and Douek 2012) where they elicit the activation of immune cells (Brenchley, Price et al. 2006, Gordon, Cervasi et al. 2010). In addition, the depletion of regulatory T cells (Eggena, Barugahare et al. 2005), the homeostatic response to lymphopenia (Catalfamo, Di Mascio et al. 2008) and the enhanced activation of pDCs along with a massive IFN-α production (Swiecki and Colonna 2010) contribute to systemic immune activation.

AIDS - EXHAUSTION OF THE IMMUNE SYSTEM CAUSES A FATAL STATE OF IMMUNODEFICIENCY

Both, the progressive depletion of $CD4^+$ T cells as well as the chronic stimulation of the immune system culminate in uncontrolled viral replication and low levels of peripheral blood $CD4^+$ T cells, disabling the immune system to control harmful or commensal microorganisms and to reject transformed cells (Douek, Roederer et al. 2009). Consequently AIDS is characterized by life-threating, opportunistic infections including pulmonary tuberculosis and a variety of viral (e.g. cytomegalovirus, adenovirus, herpes simplex virus), bacterial (e.g. *M. avium intracellulare*), parasitical (e.g. *Toxoplasma gondii*) or fungal (e.g. *Cryptococcus neoformans* and *C.albicans*) infections as well as cancers such as Kaposi sarcoma or non-Hodgkin B cell lymphomas (Pantaleo, Graziosi et al. 1993).

WHAT ALLOWS HIV-1 TO ESTABLISH A PROGRESSIVE INFECTION OF THE IMMUNE SYSTEM?

HIV-1 DISTURBS THE HOMEOSTATIC EQUILIBRIUM BETWEEN PERSISTENT VIRUSES AND THEIR HOSTS

Humans live in a symbiotic relationship with various microbes (Lisco, Vanpouille et al. 2009, Virgin, Wherry et al. 2009, Ng, Snell et al. 2013). Persistent viruses succeeded in modulating the host-virus equilibrium in a way that favors their life-long persistence for example through the induction of T cell exhaustion, by host-derived anti-inflammatory cytokine IL-10 (Brooks, Trifilo et al. 2006) or by viral latency. Thus, viral persistence can either be achieved by latency which is common for many herpes viruses such as HSV (Decman, Freeman et al. 2005, Efstathiou and Preston 2005), CMV (Barouch and Letvin 2001) or EBV (Callan 2003), or by sustained active viral replication such as HBV (Chisari and Ferrari 1995), HCV (Rehermann and Chisari 2000) and human T-lymphotrophic virus type 1 (Bangham 2000). In contrast to other persistent viral infections, HIV progressively undermines the ability of the immune system to control its replication and eventually leads to a fatal state of immunodeficiency (Douek, Roederer et al. 2009). The relatively recent introduction of HIV into humans (Hahn, Shaw et al. 2000, Sharp 2002) might also be a reason for a virus-host interaction that did not reach a stable equilibrium as found for SIV in non-human primates (Pandrea, Sodora et al. 2008). The fact that the immune system is able to mount an effective HIV-directed immune response at least in the acute phase of the infection (Borrow, Lewicki et al. 1994, Koup, Safrit et al. 1994), raises the question why the immune system ultimately fails to keep HIV under control. Undoubtedly, the systemic, pathological activation of the immune system (Douek et al, 2009; Klatt et al, 2013; Paiardini & Muller-Trutwin, 2013) as well as the capacity of HIV-1 to directly interfere with the main regulators of the adaptive immune system - the $CD4^+$ T cells (Dalgleish, Beverley et al. 1984, Klatzmann, Champagne et al. 1984, Sattentau 1988, Stevenson 2003) - are main driving forces of the progressive immunodeficiency during HIV-1 infection. In addition, HIV's potential to induce latency (Chun, Stuyver et al. 1997, Finzi, Hermankova et al. 1997, Finzi, Blankson et al. 1999, Blankson, Persaud et al. 2002, Eisele and Siliciano 2012) and its viral evolution due to the high mutation rate (Preston, Poiesz et al. 1988, Wain-

Hobson 1993, Bonhoeffer, Holmes et al. 1995, Mansky and Temin 1995) are factors that contribute to the establishment of a progressive infection.

CHRONIC IMMUNE ACTIVATION AND IMMUNODEFICIENCY ARE TWO INSEPARABLE FEATURES OF PROGRESSIVE HIV-1 INFECTION

Chronic immune activation is undoubtedly a key determining factor of the progressive immunodeficiency (Douek, Roederer et al. 2009, Klatt, Chomont et al. 2013, Paiardini and Muller-Trutwin 2013). Although a robust and strong activation of the immune system is crucial to induce protective immune responses directed against microorganism, the lack of the "off-switch" and the prolonged unspecific immune activation are not of any benefit in controlling harmful microorganisms but rather provoke the exhaustion and erosion of the immune system. The pathognomonic feature of chronic immune activation in AIDS disease progression is convincingly demonstrated by comparing SIV infected non-human primates. Despite SIV infection of natural and non-natural hosts causes similar levels of viral replication, only non-natural hosts develop substantial $CD4^+$ T cell depletion and chronic immune activation and consequently progress to an AIDS-like disease, whereas natural hosts remain healthy (Silvestri, Sodora et al. 2003, Paiardini and Muller-Trutwin 2013).

How does the chronic activation of the immune system lead to immunodeficiency? The exposure of immune cells to an inflammatory, activating milieu enhances their propensity to succumb to activation-induced cell death (AICD) (Badley, Pilon et al. 2000, Arnold, Brenner et al. 2006, Cummins and Badley 2010) and provides more substrate for viral replication based on the fact that HIV preferentially infects and replicates within activated $CD4^+$T cells (Bukrinsky, Stanwick et al. 1991) and accordingly drives disease progression. Otherwise, the progressive loss of immune control promotes the reactivation of persistent microorganisms which leads to the activation of the immune system in an attempt to counteract their reactivation (Wherry, Blattman et al. 2003, Lisco, Vanpouille et al. 2009, Virgin, Wherry et al. 2009, Naeger, Martin et al. 2010). Furthermore, the fact that the majority of abortively infected $CD4^+$ T cells die by caspase-1-mediated pyroptosis causes the release of inflammatory signals (Doitsh, Galloway et al. 2014).

DIRECT INTERFERENCE WITH CD4+ T CELL FUNCTION IS A VERY UNIQUE FEATURE OF HIV-1

HIV's potential to directly interfere with the very same cells which elicit and coordinate the immune response required for its elimination is a very unique feature of HIV. So far only human herpes virus-7 (HHV-7) is known to mediate viral entry in a CD4 receptor dependent manner (Lusso, Secchiero et al. 1994). The underlying molecular basis is the HIV-1's surface envelope glycoprotein gp120 which binds with very high affinity to the CD4 receptor (Myszka, Sweet et al. 2000). This interaction consequently causes a quantitative (Hazenberg, Hamann et al. 2000, McCune 2001, Grossman, Meier-Schellersheim et al. 2002) and qualitative (Clerici, Stocks et al. 1989) decline of $CD4^+$ T cells and thereby represent two fundamentally distinct mechanisms contributing to $CD4^+$ T cell dysfunction in HIV-1 infections. Such specific targeting of central and sensitive regulators of the immune system is a crucial factor for the progressive immunodeficiency which is associated with increased susceptibility to opportunistic infections, since it impairs $CD4^+$ T cell help and therefore optimal humoral (Parker 1993, Oxenius, Zinkernagel et al. 1998) as well as cellular (Shedlock and Shen 2003) adaptive immune responses. In line with that, the importance of $CD4^+$ T cells in coordinating an anti-viral immune response is provided from studies of SIV infected macaques in which $CD4^+$ T cell depletion causes an accelerated disease progression (Ortiz, Klatt et al. 2011). Furthermore, suppression of HIV-1 replication by ART is associated with a recovery of $CD4^+$ T cells which brings along a significant recovery of overall immune function (Wei, Ghosh et al. 1995, Autran, Carcelain et al. 1997). The progressive decline of $CD4^+$ T cells is generally attributed to a direct cytopathic effect (Lenardo, Angleman et al. 2002) in activated, productively infected cells or to pyroptosis of resting, abortively infected cells (Doitsh, Galloway et al. 2014). A direct cytopathic effect as sole cause for the progressive decline of $CD4^+$ T cells was brought into question since the level of $CD4^+$ T cell loss is disproportionally high in comparison to the levels of infectious virus and productively infected cells (Harper, Marselle et al. 1986, Haase, Henry et al. 1996, Chun, Carruth et al. 1997, Anderson, Ascher et al. 1998, Haase 1999, Douek, Picker et al. 2003) and consequently bystander mechanisms play a crucial role in mediating dysfunction and loss of non-HIV-infected nor HIV-specific $CD4^+$ T cells (Harper, Marselle et al. 1986, Douek, Brenchley et al. 2002). Bystander effects involve increased expression of death receptors Fas/FasL (Oyaizu, McCloskey et al. 1994, Mitra, Steiner et al. 1996, Sloand, Young et al. 1997, Kaplan and

Sieg 1998) or TRAIL/DR5 (Lichtner, Maranon et al. 2004, Herbeuval, Grivel et al. 2005, Herbeuval and Shearer 2007), increased propensity of AICD (Badley, Pilon et al. 2000, Arnold, Brenner et al. 2006, Cummins and Badley 2010) and the induction of dysfunction in the face of chronic immune activation (Douek, Roederer et al. 2009) due to exposure to microbial products and inflammatory cytokines (Brenchley, Price et al. 2006).

AIM OF THE THESIS

The progressive decline in the number and function of $CD4^+$ T cells is a hallmark of progression to AIDS. High amounts of gp120 - mostly in the form of gp120-anti-gp120 ICs - are present in the plasma and lymphoid tissues from HIV-1 patients and since gp120 has a very high affinity to the CD4 receptor, the gp120-CD4 receptor interaction is considered to play a central role during HIV-1 infection. It's functional outcome however, specifically in the context of TCR-induced $CD4^+$ T cell activation, is poorly understood and contradictory results have been reported in the literature. In this thesis we investigated how HIV-1 gp120 in its soluble form affects the function of $CD4^+$ T cells during HIV-1 infection.

In a first part, to consolidate the reported controversies with respect to activatory and inhibitory signals conveyed by non-infectious gp120, we set up *in vitro* experiments to investigate in detail how gp120 or gp120-anti-gp120 ICs interfere with $CD4^+$ T cell activation upon stimulation either with agonistic anti-human CD3 mAbs to obtain maximum level of stimulation or with HLA class II presented peptide antigens for a more physiological readout. We demonstrate that spatially and temporally linked CD4 and TCR triggering at a defined site by plate immobilized stimuli promotes full $CD4^+$ T cell activation by exceeding local thresholds for signaling propagation. On the contrary, when $CD4^+$ T cells were co-cultured with gp120-anti-gp120 IC loaded APCs, these ICs were quantitatively transferred to $CD4^+$ T cells, resulting in strongly impaired responsiveness towards TCR stimulation induced by agonistic anti-human CD3 mAbs or peptide loaded MHC molecules.

In a second part, we investigated the mechanistic basis for these discrepant outcomes of gp120-CD4 receptor interaction and show that spatially confined TCR and CD4 engagement promoted immunological synapse (IS) formation and hence proximal TCR signaling, whereas gp120-anti-gp120 IC bound to CD4 receptors on the entire surface of responding $CD4^+$ T cells inhibited the cytoskeletal rearrangement required for establishment of the IS.

In a last part, we investigate a potential *in vivo* relevance of the aforementioned scenarios and show that $CD4^+$ T cells from HIV^+ individuals are covered with ICs which contain gp120. We suggest that gp120-

containing IC binding to CD4 receptors might contribute to the progressing decline of $CD4^+$ T cell function during HIV-1 infection.

Results

HIV-1 GP120-ANTI-GP120 IMMUNE COMPLEX MEDIATED MODULATION OF CD4+ T CELL FUNCTION

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KZ and AO designed the experiments. KZ, AH and MR performed experiments and analyzed data. KZ and AO discussed data. AT contributed anti-gp120 antibodies. KZ and AO made the figures and wrote the manuscript.

SUMMARY

Besides virion associated and cell expressed HIV-1 envelope glycoprotein, considerable amounts of soluble gp120 and gp160 are found in the plasma and lymphoid tissues of HIV-1 patients. Free gp120 retains a high affinity for the CD4 receptor, but the consequence of gp120 binding to CD4+ T cells is poorly understood. We therefore performed *in vitro* experiments to address the role of circulating gp120 (and gp120-anti-gp120 immune complexes (IC)) in HIV-associated CD4+ T-cell dysfunction. We demonstrate that gp120 strongly interferes with T cell receptor (TCR) signaling efficiency and thereby modulates CD4+ T cell activation. Gp120's potential to either enhance or diminish CD4+ T cell activation depends on the relative orientation of TCR versus CD4 receptor engagement, whereas a positive effect of gp120 or gp120-anti-gp120 ICs was observed in a rather "artificial" situation in which stimuli are immobilized on a planar substrate, gp120 ICs are adsorbed to CD4 receptors on CD4+ T cells in a more physiological assay and thereby impair the TCR-induced activation of these cells by undermining proximal positive signaling beyond the engaged TCRs.

INTRODUCTION

The fact that the HIV-1 surface envelope glycoprotein gp120 renders CD4+ T cells prime targets for infection (Dalgleish, Beverley et al. 1984, Sattentau 1988) via the high affinity gp120-CD4 receptor interaction (Myszka, Sweet et al. 2000) might contribute to the characteristic progressive decline of CD4+ T cell function during HIV-1 infection. Interference with the very same cells that also coordinate the elimination might represent a key mechanism in HIV-1's ability to evade functional immune responses, since it undermines CD4+ T cell mediated help for the induction of humoral (Parker 1993, Oxenius, Zinkernagel et al. 1998) as well as cellular (Shedlock and Shen 2003) adaptive immune responses. The fact that quantitative (Hazenberg, Hamann et al. 2000, McCune 2001, Grossman, Meier-Schellersheim et al. 2002) and qualitative (Clerici, Stocks et al. 1989) defects within the CD4+ T cell population in HIV-1 infected individuals are disproportionally high in comparison to the levels of infectious virus and productively infected cells (Harper, Marselle et al. 1986, Douek, Picker et al. 2003), attributes CD4+ T cell directed viral cytopathicity a minor contributor to disease progression. However, HIV-1 replication, which is fundamental to disease progression, is not only associated with virion associated envelope glycoprotein but also with soluble gp120 and gp160 due to shedding from the virus or from infected cells (Gelderblom, Reupke et al. 1985, Schneider, Kaaden et al. 1986, Pyle, Bess et al. 1987). Consequently, soluble gp120 is present in the plasma (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Rychert, Strick et al. 2010) or lymphoid tissues of HIV+ patients (Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009), and together with the appearance of dysfunctional CD4+ T cells, it is conceivable that binding of soluble gp120 to CD4+ T cells may be a central parameter in HIV-1 pathogenesis what could at least partially explain the high proportion of dysfunctional CD4+ T cells.

CD4 receptors play a crucial role in enhancing the sensitivity of TCR-triggered CD4+ T cell activation by interacting with MHC II molecules on antigen-presenting cells (APC) (Gay, Maddon et al. 1987, Glaichenhaus, Shastri et al. 1991) and by their non-covalent interaction with the src-family ymphocyte cell-specific protein-tyrosine kinase (p56lck) whose activation initiates TCR signaling progression (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991). Going in line with that, a plethora of *in vitro* studies provides experimental proof that gp120 binding to the CD4 receptor interferes with TCR-induced CD4+ T cell activation (Chirmule, Kalyanaraman et al.

1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Oravecz and Norcross 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009). However, the effect of non-infectious gp120-CD4 receptor interaction on CD4+ T cell activation is still a contradictory issue in the literature. On the one side, gp120 interaction with CD4 receptors was suggested to enhance the activation of CD4+ T cells in terms of increased calcium signaling and IL-2R expression (Kornfeld, Cruikshank et al. 1988), transiently enhanced p56lck activity (Juszczak, Turchin et al. 1991, Hivroz, Mazerolles et al. 1993), activation of the transcription factors AP-1 (Chirmule, Goonewardena et al. 1995) and elevated proliferation (Oravecz and Norcross 1993), and on the other side, gp120 binding to CD4+ T cells was shown to reduce their activation mediated through TCR stimulation (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Cefai, Ferrer et al. 1992, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009).

Based on these controversial reports, we investigated in this study if and how non-virion associated gp120 interferes with TCR-induced CD4+ T cell activation. With the aim to mimic the *in vivo* situation as close as possible, we performed our *in vitro* experiments with primary CD4+ T cells and gp120-anti-gp120 ICs which represent the most predominant *in vivo* form of non-virion associated gp120 (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1996). To verify the impact of gp120 on anti-CD3 mAb- or peptide -induced CD4+ T cell activation, their activation was analyzed in the presence of absence of additional CD4 receptor cross-linking by gp120 or gp120-anti-gp120 ICs. Our study revealed that gp120-anti-gp120 ICs were rapidly and quantitatively transferred from APCs to CD4+ T cells and importantly, during this process cross-linked CD4 receptors proved to substantially prevent subsequent TCR-mediated activation of CD4+ T cells. In contrast, full CD4+ T cell activation was promoted if CD4 and TCR cross-linking was provided in close proximity by immobilized gp120 and anti-human CD3 mAb on a planar substrate.

RESULTS

Plate immobilized gp120 enhances TCR-induced activation of CD4+ T cells

CD4 receptors play an essential role in TCR-mediated activation of CD4+ T cells through their intracellular association with the T cell specific kinase p56lck (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991). Since gp120 binds with high affinity to the CD4 receptor (Myszka, Sweet et al. 2000), a potential modulating capacity of TCR-driven CD4+ T cell activation is attributed to gp120. Based on the importance of CD4+ T cells as central players of the adaptive immune system and the high availability of soluble gp120 in the plasma or lymphoid tissue of HIV-1 patients (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009, Rychert, Strick et al. 2010), we set out to investigate how non-virion associated gp120 affects TCR-induced CD4+ T cell activation. In a first *in vitro* assay, CD4+ T cells from healthy donors were stimulated with plate immobilized recombinant gp120 in combination with low amounts (50 ng/well) of agonistic anti-human CD3 mAb, which we previously determined to induce only marginal CD4+ T cell activation by itself. In combination however, simultaneous CD4 receptor/suboptimal TCR cross-linking by plate immobilized gp120/anti-human CD3 mAb significantly enhanced CD4+ T cell activation compared to gp120 and anti-CD3 mAb stimulation alone. Activation was determined by the expression of early T cell activation markers CD69 and CD40L within 6 hours (Figure 1A - 1C) and levels of proliferation were measured by CFSE dilution after 5 days (Figure 1D and 1E). Importantly, CD4 receptor cross-linking by gp120 in the absence of concomitant TCR stimulation did not have any activating capacity on CD4+ T cells (Figure 1A and 1B). These data suggest that CD4 receptor cross-linking quantitatively supports TCR-induced signaling, yielding full CD4+ T cell activation if signals are provided in close temporal and spatial proximity. As opposed to activation marker expression and proliferation, IL-2 production critically depends on TCR signaling in combination with an additional signal coming from a separate co-stimulatory receptor such as CD28 (Schmitz and Krappmann 2006). Going in line with a selective role of CD4 receptors in reinforcing proximal TCR signaling, IL-2 production was not induced by gp120 mediated CD4 receptor cross-linking (Figure 1F).

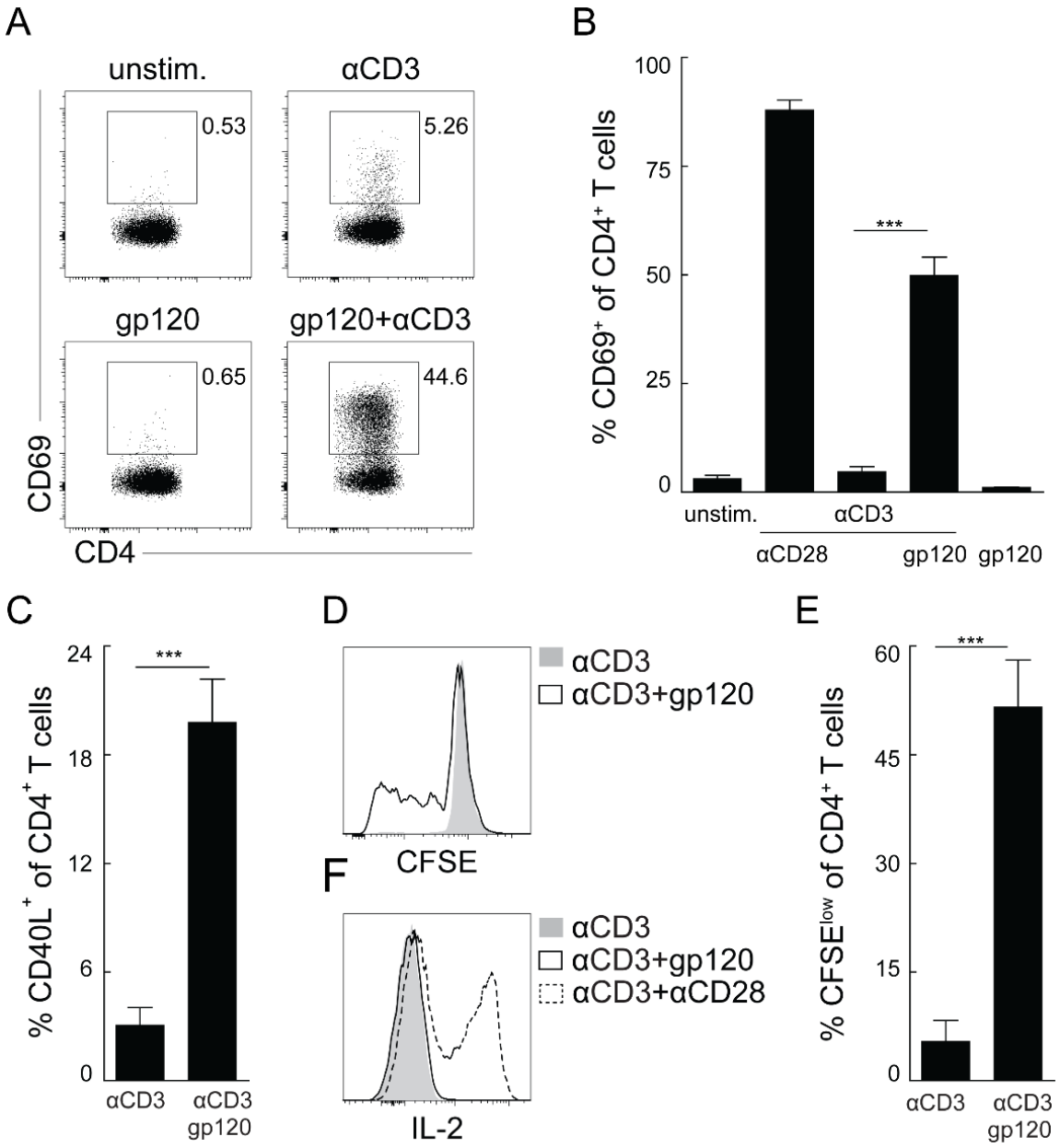

Figure 1: Plate immobilized gp120 increases TCR-induced activation of CD4+ T cells.

CD4+ T cells from healthy donors were stimulated for 6 hours (A-C and F) or 5 days (D and E) with sub-activating amounts of plate immobilized anti-human CD3 mAb in the presence or absence of gp120. As control, CD4+ T cells were similarly stimulated with anti-human CD3 mAb in combination with anti-human CD28 mAb, with gp120 alone or they were left untreated. (A) Dot plot of one representative experiment showing CD69 expression on CD4+ T cells. (B) Bar graph depicting average expression levels of CD69 on CD4+ T cells (n = 14, bars represent mean ± SEM, ***p < 0.001, one-tailed, paired t-test). (C) Bar graph illustrates the expression of CD40L on CD4+ T cells (n = 6, bars represent mean ± SEM, ***p < 0.001, one-tailed, paired t-test). (D) Representative flow cytometry histogram showing CFSE dilution profile of CD4+ T cells. (E) Bar graph represents the average frequency of CFSElow CD4+ T cells (n = 6, bars represent mean ± SEM, ***p < 0.001, one-tailed, paired t-test). (F) Flow cytometry histogram depicts IL-2 expression in CD4+ T cells. n = nr of individual donors.

Gp120-induced increase of CD4+ T cell activation is more efficient for memory than for naïve CD4+ T cells

In a next step, we investigated whether naïve and memory CD4+ T cells are equally responsive to gp120-induced increase of CD4+ T cell activation. Therefore, peripheral CD4+ T cells from healthy donors were sorted into naïve and memory cells according to CD45RA and CD11a expression (whereas CD45RA+ CD11a^{dim} CD4+ T cells were considered as naïve and CD45RA- CD11a^{bright} CD4+ T cells as memory cells). Accordingly, CD4+ T cells were stimulated with plate-immobilized low amounts of agonistic anti-human CD3 mAb alone or in combination with recombinant gp120. The gp120-mediated increase of CD4+ T cell activation was more efficient for memory than for naïve cells (Figure 2), what might reflect varying requirements for T cell activation within naïve or memory T cells (Kuiper, Brouwer et al. 1994).

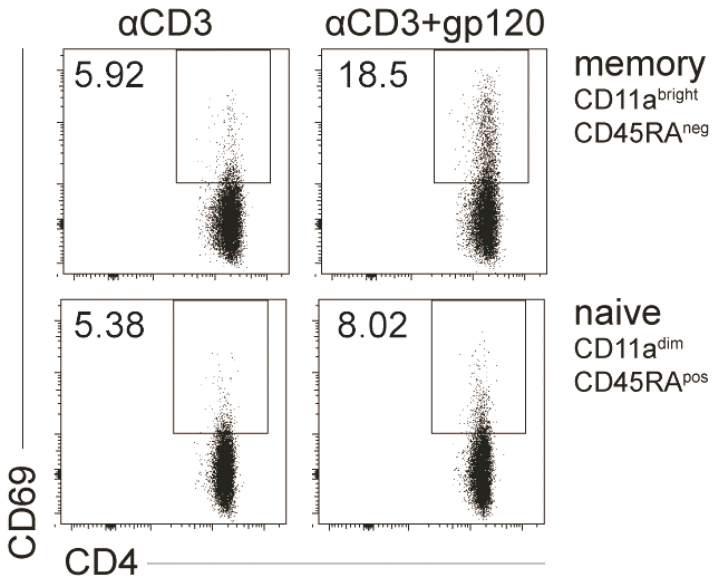

Figure 2: Gp120 enhanced CD4+ T cell activation is more efficient for memory than for naïve CD4+ T cells.

Memory and naïve CD4+ T cells from healthy donors were stimulated for 6 hours with sub-activating amounts of plate immobilized anti-human CD3 mAb in the presence or absence of gp120. Dot plots of one representative experiment showing CD69 expression on naïve CD45RA+ CD11a^{dim} and memory CD45RA+ CD11a^{dim} CD4+ T cells.

Gp120- or anti-CD4 mAb mediated CD4 cross-linking enhances CD4+ T cell activation if provided in temporal proximity to the TCR engagement

The potent capacity of HIV-1 gp120 to modulate TCR-induced CD4+ T cell activation in a positive manner, raises the question whether full CD4+ T cell activation is induced if gp120 and low amounts of anti-CD3 mAbs are provided in a temporally separated manner. Such temporal separation of CD4 and TCR engagement was achieved by incubating CD4+ T cells consecutively on a gp120- and anti-CD3 mAb-coated well. Analysis of CD4+ T cell activation revealed that transient, precedent CD4 receptor crosslinking by gp120 stimulation did not strengthen subsequent TCR stimulation (Figure 3A). To assess whether the

stimulatory effect of gp120, when provided simultaneously with the TCR stimulus was an intrinsic feature of gp120, we also tested the ability of CD4 cross-linking antibodies to enhance stimulation of CD4+ T cells. Indeed, concomitant anti-CD4 mAb mediated cross-linking of the CD4 receptor lead to full CD4+ T cell activation in the presence of low levels of anti-CD3 mAbs, indicating that any CD4 cross-linking modality would promote CD4+ T cell activation when provided in a temporally and spatially linked manner (Figure 3B).

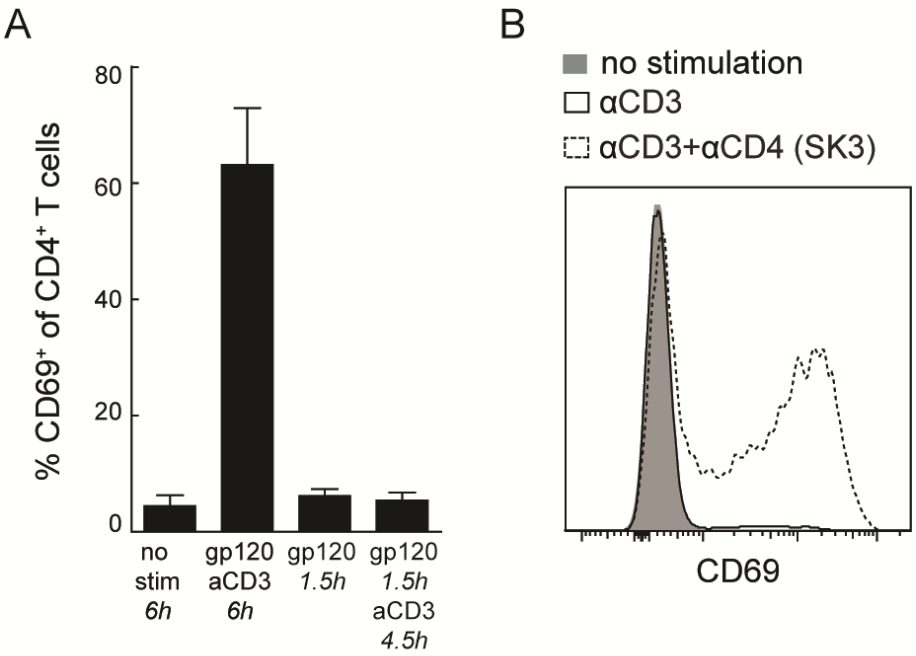

Figure 3: Gp120 enhances CD4+ T cell activation in a non-intrinsic manner if provided in temporal proximity.

(A) CD4+ T cells from healthy donors were stimulated on wells with plate-immobilized gp120 for 1.5 hours and subsequently transferred to empty wells or wells coated with sub-activating amounts of plate immobilized anti-human CD3 mAb for another 4.5 hours. As a control, CD4+ T cells were stimulated simultaneously on gp120 / α CD3 mAb co-coated wells for 6 hours or left unstimulated (n = 3, bars represent mean ± SEM). (B) CD4+ T cells from healthy donors were stimulated for 6 hours with sub-activating amounts of plate immobilized anti-human CD3 mAb in the presence or absence of an anti-CD4 mAb (clone SK3, 1µg / well) or were left untreated. Representative flow cytometry histogram depicts CD69 expression on CD4+ T cells.

Gp120-mediated increase of CD4+ T cell activation depends on gp120 binding to the CD4 receptor

The capacity of gp120 to selectively enhance TCR-induced activation of human (Figure 1) and not mouse CD4+ T cells (Figure 4A) whose CD4 receptor does not bind gp120 (McClure, Sattentau et al. 1987), indicates that gp120-CD4 receptor interaction is crucial for gp120-mediated enhancement of CD4+ T cell activation and also excludes the possibility that impurities of the gp120 preparation influenced the observed CD4+ T cell activation. To substantiate the prerequisite of gp120-CD4 receptor interaction in promoting CD4+ T cell activation, we used in a next step antibodies which specifically blocked the gp120 binding site on the CD4 receptor (Sattentau, Dalgleish et al. 1986). As expected, gp120 binding to CD4+ T cells was reduced to background levels in the presence of the CD4 receptor blocking antibody (clone SK3) (Figure 4B and 4C) and such blocking of the gp120 binding site on the CD4 receptor selectively abrogated αCD3/gp120- but not αCD3/αCD28-induced activation of CD4+ T cells (Figure 4D). Furthermore, CD8+CD4+ T cells, which are present in the blood at low frequencies (Kitchen, Korin et al. 1998), were efficiently activated by gp120 in the presence of low amounts of anti-human CD3 mAb, whereas this was not the case for CD8+CD4- T cells (Figure 4E).

The CD4 receptor associated p56lck is crucial for proximal TCR signaling (Rudd, Anderson et al. 1989, Glaichenhaus, Shastri et al. 1991, Zamoyska, Basson et al. 2003) by initiating a sequential cascade of tyrosine phosphorylation on signaling molecules, as for example on the linker of activated T cells (LAT) which is the nucleating site for multiprotein signaling complexes (Balagopalan, Coussens et al. 2010). We found that simultaneous CD4 receptor/TCR cross-linking by plate immobilized gp120 and low amounts of plate immobilized anti-human CD3 mAb induced slightly elevated levels of phosphorylated LAT, similar as under CD28 co-stimulatory conditions, in relation to CD4+ T cells which received weak TCR stimulation alone (Figure 4F and 4G).

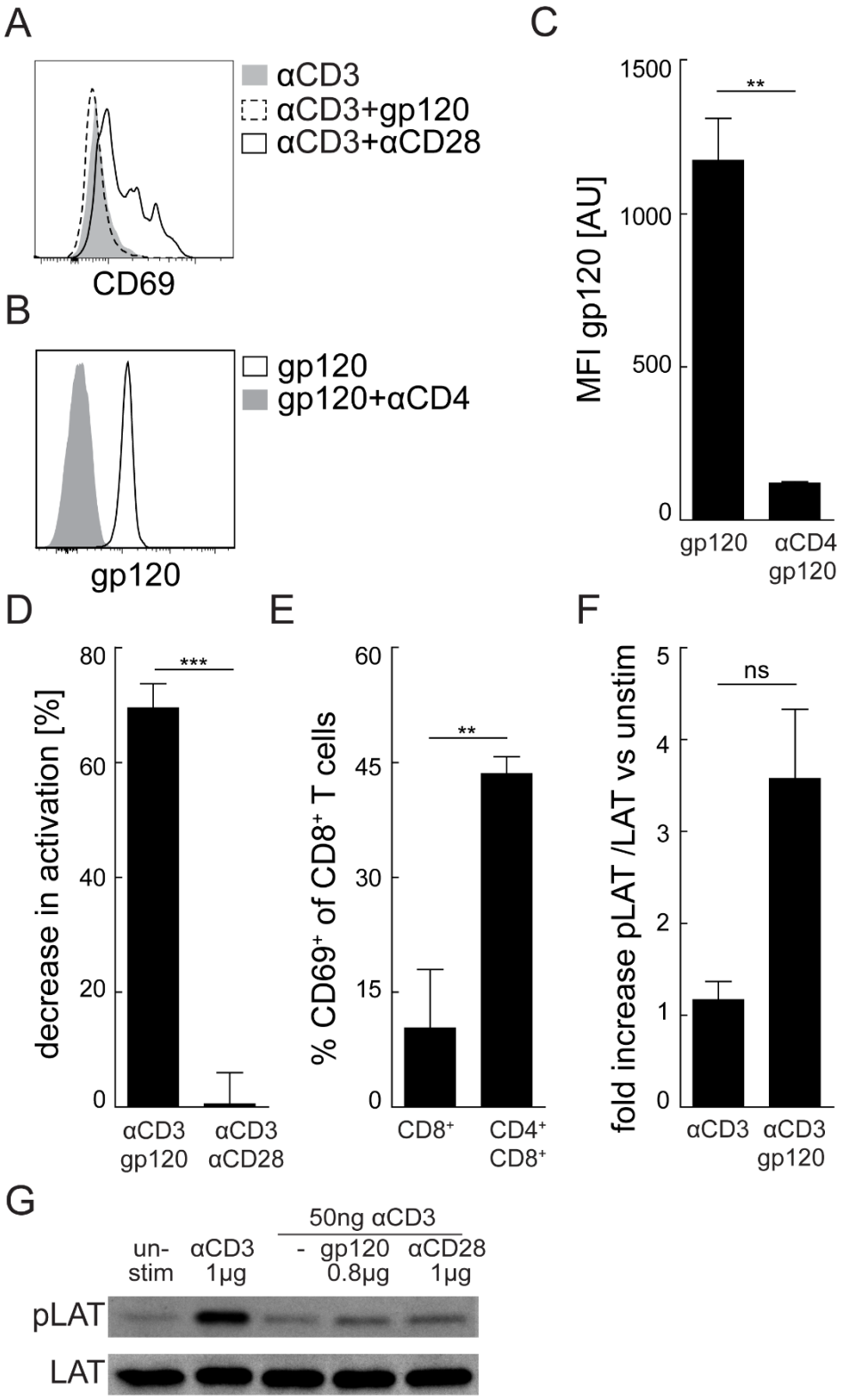
A
αCD3
αCD3+gp120
αCD3+αCD28
CD69
B
gp120
gp120+αCD4
gp120
C
MFI gp120 [AU]
1500
1000
500
0
**
gp120
αCD4 gp120
D
decrease in activation [%]
80
60
40
20
0
***
αCD3 gp120
αCD3 αCD28
E
% CD69+ of CD8+ T cells
60
45
30
15
0
**
CD8+
CD4+ CD8+
F
fold increase pLAT /LAT vs unstim
5
4
3
2
1
0
ns
αCD3
αCD3 gp120
G
50ng αCD3
un-stim
αCD3 1µg
- gp120 0.8µg
αCD28 1µg
pLAT
LAT

Figure 4: TCR-mediated activation of CD4+ T cells is enhanced significantly in the presence of CD4 receptor engagement by plate immobilized gp120.

(A) Flow cytometry histogram illustrates CD69 expression on mouse CD4+ T cells after stimulation with plate immobilized anti-mouse CD3 mAb alone or in combination with gp120 or anti-mouse CD28 mAb for 6 hours. (B-D) CD4+ T cells isolated from HIV-1- human donors were pre-incubated with anti-human CD4 mAb (clone SK3) to block the gp120 binding site on the CD4 receptor. (B) Representative flow cytometry histogram showing gp120 binding on CD4+ T cells ± αCD4. (C) Bar graph depicting MFI signal in AU of gp120 on CD4+ T cells ± αCD4 (n = 3, bars represent mean ± SEM, **p < 0.01, one-tailed, paired t-test). (D) Bar graph depicting the relative decrease in activation induced by stimulation with plate immobilized αCD3+gp120 or αCD3+αCD28 in the presence versus absence of αCD4 (measured by CD69 expression after 6 hours) (n = 9, bars represent mean ± SEM, ***p < 0.001, one-tailed, paired t-test). (E) Bar graph summarizes the expression of CD69 on human $CD8^+CD4^-$ (n = 3) or $CD8^+CD4^+$ (n = 2) T cells stimulated with plate immobilized αCD3 ± gp120 for 6 hours (bars represent mean ± SEM, **p < 0.01, one-tailed, unpaired t-test). (F - G) CD4+ T cells isolated from HIV-1- human donors were stimulated for 2-10 minutes with plate immobilized anti-human CD3 mAb alone or in combination with gp120 / anti-human CD28 mAb or they were left unstimulated. (F) Bar graph indicates the average fold increase of phospho-LAT/LAT levels (n = 3, bars represent mean ± SEM, ns, not significant p=0.0524, one-tailed, paired t-test). (G) Corresponding representative immunoblot showing the levels of phosphorylated and unphosphorylated LAT. n = nr of individual donors.

These experiments support the notion that gp120 binding to the CD4 receptor not only represents the first step of viral entry, but in addition has the potential to significantly increase CD4+ T cell activation by quantitatively supporting proximal TCR signaling.

Gp120-anti-gp120 ICs activate CD4+ T cells in a comparable manner to monomeric gp120

Since gp120 (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009, Rychert, Strick et al. 2010) as well as anti-gp120 antibodies (Lyerly, Reed et al. 1987, Doria-Rose, Klein et al. 2009, McCoy and Weiss 2013) are present in HIV-1 patients, gp120-anti-gp120 ICs are likely to be generated and abundant *in vivo* (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1996). We therefore generated such gp120-anti-gp120 ICs by co-incubating recombinant gp120 with two different human IgG anti-gp120 mAb at a molar gp120 to anti-gp120 mAb ratio of approximately 4 to 5. As schematically shown in Figure 5A, either two different anti-gp120 mAbs that block the CD4 binding site (CD4BS) (clone b6 (Pantophlet, Ollmann Saphire et al. 2003) and b12 (Barbas, Bjorling et al. 1992, Burton, Pyati et al. 1994)) or anti-gp120 mAbs that spare the CD4 binding site (non-CD4BS) (clone 2G12 (Trkola, Purtscher et al. 1996) and 1-79 (Scheid, Mouquet et al. 2009)) were used for the formation of gp120-anti-gp120-CD4BS ctrl IC (left) or gp120-anti-gp120 IC (right), which are abbreviated as ctrl IC or

IC respectively from here on. Consequently, the ctrl ICs which contain anti-gp120 antibodies that target the CD4 binding site do not allow any interaction with the CD4 receptor and are therefore not expected to have any functional effect on CD4+ T cells. Our results clearly show that similarly to non-virion associated gp120 alone, plate immobilized IC significantly enhanced CD4+ T cell activation induced by weak TCR stimulation, as measured by CD69 expression (Figure 5B and 5C) after 6 hours. In contrast, the ctrl IC failed to do so (Figure 5B and 5C). In addition, proliferation of CD4+ T cells determined by CFSE dilution as well as the expression of late T cell activation markers CD25 and CD38 were significantly increased in the presence of IC compared to the ctrl IC in combination with low level of TCR stimulation (Figure 5D and 5E) after 5 days. Thus, gp120 within the context of ICs enhances low level of TCR-driven CD4+ T cell activation in a CD4 receptor dependent manner similar to gp120 alone.

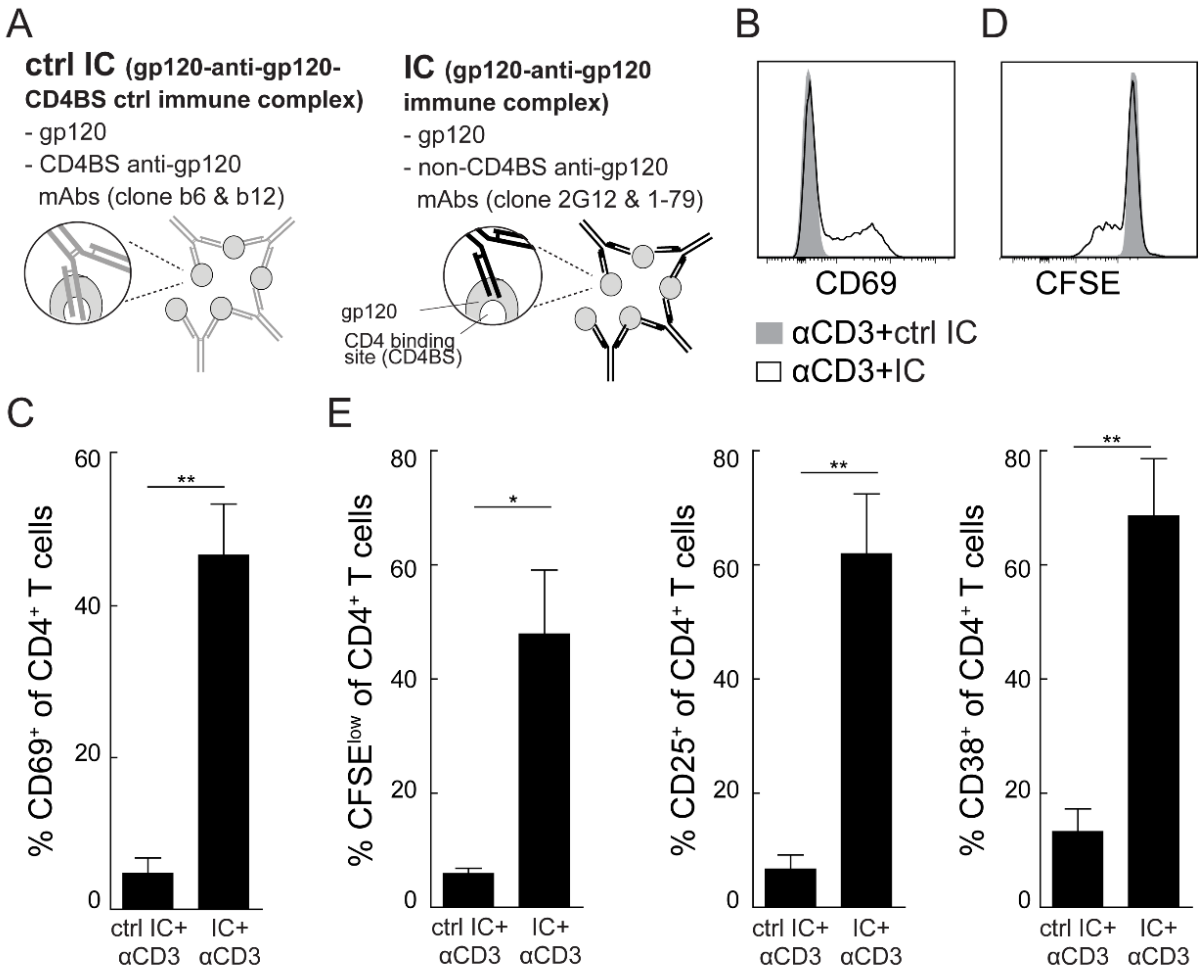

Figure 5: Plate immobilized gp120-anti-gp120 ICs augment TCR-induced CD4+ T cell activation in a CD4 receptor dependent manner.

(A) Schematic representation of gp120-anti-gp120-CD4BS ctrl IC (ctrl IC; left) and gp120-anti-gp120 IC (IC; right) consisting of gp120 in combination with either two CD4BS anti-gp120 mAbs (clone b6 and b12) or non-CD4BS anti-gp120 mAbs (clone 2G12 and 1-79) at a molar gp120:ab ratio of ~4:5. CD4+ T cells were stimulated for 6 hours (B and C) or for 5 days (D and E) with plate immobilized ctrl IC or IC in the presence of anti-human CD3 mAb. (B) Representative flow cytometry histogram showing CD69 expression on CD4+ T cells. (C) Bar graph depicting CD69 expression on CD4+ T cells (n = 4, bars represent mean ± SEM, **p < 0.01, one-tailed, paired t-test). (D) Representative CFSE dilution profile of CD4+ T cells. (E) Bar graphs showing % of $CFSE_{low}$, CD25+ and CD38+ CD4+ T cells (n = 5, bars represent mean ± SEM, *p < 0.05, **p < 0.01, one-tailed, paired t-test). n = nr of individual donors.

The amount of human IgG on CD4+ T cells was increased upon incubation with gp120 that had previously been complexed with two different anti-gp120 mAbs compared to CD4+ T cells that were incubated with gp120 that had only been complexed with an anti-gp120 mAb of a single specificity. Of note, total anti-gp120 mAbs concentrations were comparable in both situations (Figure 6). The increased amount of human IgG staining on the surface of CD4+ T cells is indicative of ICs whose formation is only possible if two different antibodies complex a given protein.

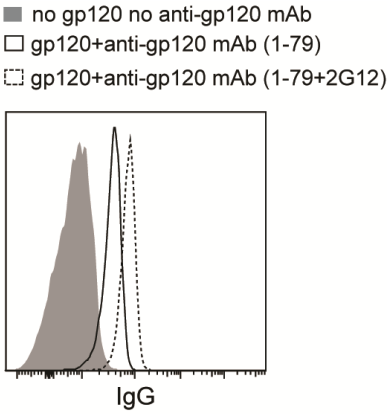

Figure 6: Increased levels of human IgG on CD4+ T cells upon incubation with gp120 that had been complexed with two different compared to one anti-gp120 mAb.

CD4+ T cells were incubated with recombinant gp120 that was pre-incubated with either one (clone 1-79, f.c. 10 µg/ml) or two (clone 1-79 and 2G12, f.c. 5 µg/ml each) anti-gp120 mAbs. IC formation was investigated by anti-human IgG binding on CD4+ T cells. Representative flow cytometry histogram depicts the level of IgG on CD4+ T cells.

Fast transfer of monocyte-bound gp120-anti-gp120 IC to the CD4 receptor of CD4+ T cells

In a next step, we set out to investigate whether the activating potential of gp120 can be translated to a more physiological situation. To mimic a condition in which ICs made of gp120 and anti-gp120 antibodies interact with CD4 receptors during TCR-induced CD4+ T cell stimulation by APCs, CD4+ T cells from healthy donors were stimulated with autologous monocytes that had previously been incubated with gp120 containing IC as well as anti-human CD3 mAb. Initially, flow cytometric determination of IC localization was performed by the analysis of human IgG binding after 6 hours of co-culture on CD4+ T cells or CD14+

monocytes. Ctrl ICs were predominantly found on the CD14+ monocytes whereas the IC was almost exclusively found on CD4+ T cells (Figure 7A). A similar localization pattern of ctrl IC and IC was confirmed by the analysis of human IgG localization after 2 hours by confocal microscopy (Figure 7B). These experiments showed that only the IC and not the ctrl IC was efficiently transferred to the CD4+ T cells from monocytes on which they were initially localized. The cell-cell transfer of ICs proved to be very fast and efficient, as already after 60 minutes most ICs were localized on CD4+ T cells (Figure 7C). Furthermore, the transfer of ICs to CD4+ T cells was abrogated in the presence of an anti-human CD4 mAb which blocks the gp120 binding site on the CD4 receptor (Figure 7D and 7E).

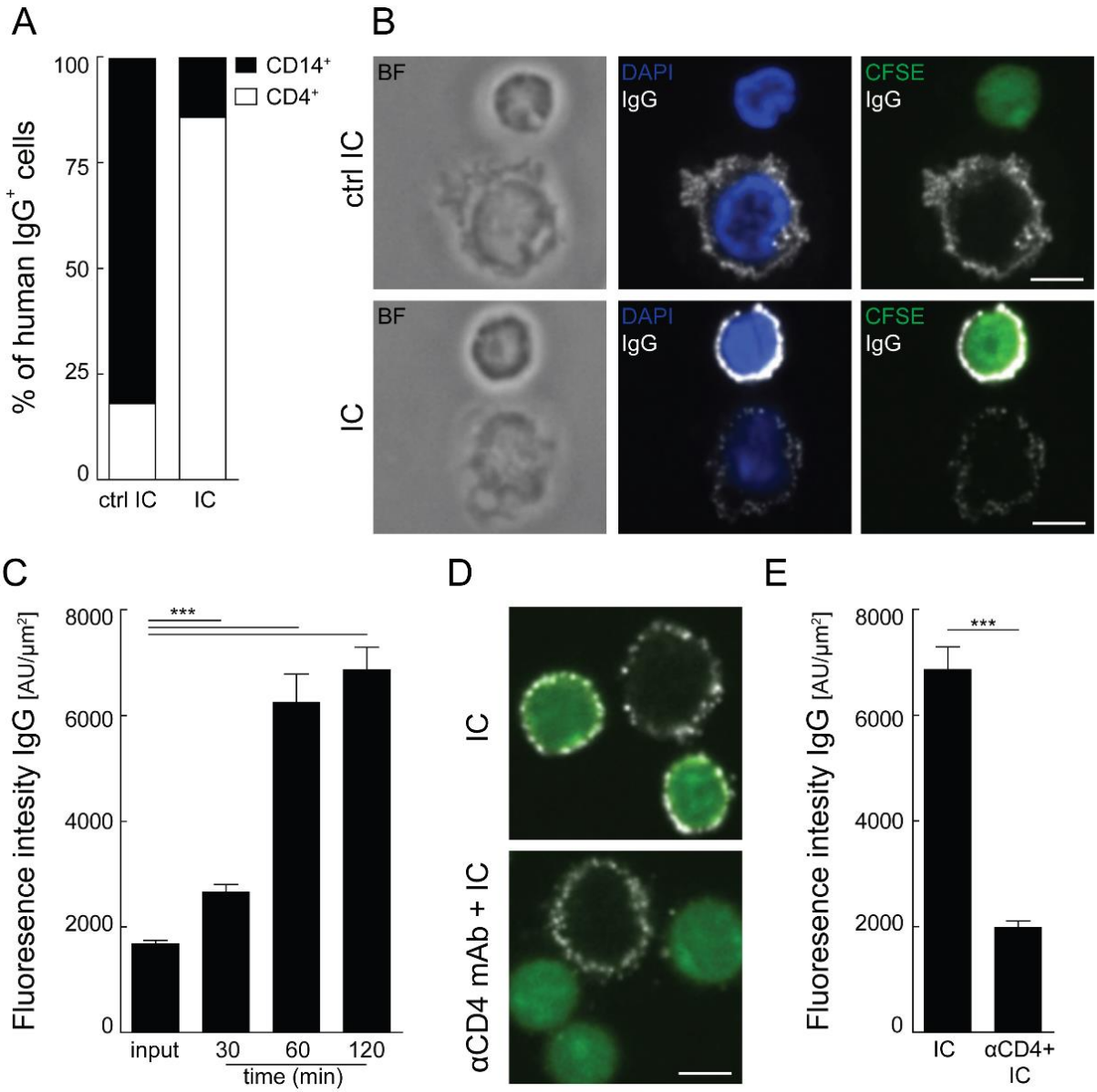

Figure 7: Gp120-anti-gp120 ICs are transferred from monocytes to CD4+ T cells in a CD4 receptor dependent manner.

CFSE-labeled CD4+ T cells were co-cultured for 6 hours (A), 2 hours (B, D and E) or various time points (C) with ctrl IC or IC loaded CD14+ monocytes. CD4+ T cells were pre-incubated with an anti-human CD4 mAb (clone SK3) to block the gp120 binding site on the CD4 receptor if specified. (A) Bar graph represents % of CD4+ T cells (white) and CD14+ monocytes (black) which stain for human IgG as measure for ctrl IC and IC (as determined by flow cytometry). (B) Representative confocal image showing anti-human IgG for visualization of IC (in white) on CFSE-labeled CD4+ T cells (green) and CD14+ monocytes, nuclear DNA is visualized by DAPI staining (blue). (C) Bar graph represents quantification of fluorescence intensity of anti-human IgG (in AU/µm^2) as measure of IC on CD4+ T cells after 0 (input, n = 17), 30 (n = 25), 60 (n = 12) and 120 (n = 24) minutes of co-culture with IC loaded monocytes (bars represent mean ± SEM, ***p < 0.001, one-tailed, unpaired t-test). (D) Representative confocal image of anti-human IgG staining (white) ± α CD4. (E) Bar graph represents quantification of fluorescence intensity of anti-human IgG (in AU/µm^2) as measure for IC on CD4+ T cells in the presence (n = 24) and absence (n = 22) of α CD4 (bars represent mean ± SEM, ***p < 0.001, one-tailed, unpaired t-test). n = number of individual cells, scale bar = 5µm.

TCR-induced CD4+ T cell activation is impaired in the presence of CD4 receptor cross-linking by gp120-anti-gp120 IC

We next investigated whether ICs, which were transferred from monocytes to CD4 receptors on CD4+ T cells, would also reinforce responsiveness towards TCR stimulation induced by anti-human CD3 mAb coated monocytes, as it was the case if the TCR and CD4 receptor were cross-linked in close proximity at a defined site as for example upon immobilization of the stimuli on a plate (Figure 1 - 6).

To this end, CD4+ T cells were stimulated with monocytes that had previously been loaded with ctrl IC and IC in combination with anti-human CD3 mAb. Interestingly, ICs that were transferred to CD4+ T cells and consequently not only cross-linked CD4 receptors at the site of TCR engagement but on the whole CD4+ T cell surface, impaired CD4+ T cell activation induced by anti-human CD3 mAb loaded monocytes in comparison to control conditions with ctrl IC loaded monocytes. The reduction was manifested by the expression of CD69 after 6 hours (Figure 8A) or CD25 and CD38 expression as well as proliferation measured by CFSE dilution after 5 days (Figure 8B and 8C). Of importance, the inhibitory effect of IC on TCR-induced CD4+ T cell activation was not limited to polyclonal activation induced by anti-human CD3 mAb but also held true for CMV-specific CD4+ T cell activation induced by HLA class II bound CMV peptides on monocytes, as the level of CD25 and CD38 expression as well as proliferation of CMV-specific CD4+ T cells was significantly reduced in the presence of IC compared to ctrl IC (Figure 8B and 8C). The fact that IC coated CD4+ T cells were still susceptible to mitogenic stimulation with PMA and ionomycin (Figure 8D)

implies that IC interferes with a proximal TCR signaling event rather than rendering CD4+ T cells completely incompetent to respond to activating stimuli in general.

Taken together, our data indicate that the way of CD4 receptor cross-linking by gp120 dictates in which way TCR-driven CD4+ T cell is affected. Whereas simultaneous TCR/CD4 receptor cross-linking confined to a specific site leads to enhanced CD4+ T cell activation, CD4 receptor cross-linking by IC not only at the site of TCR engagement manifested impaired responsiveness towards TCR-induced CD4+ T cell activation.

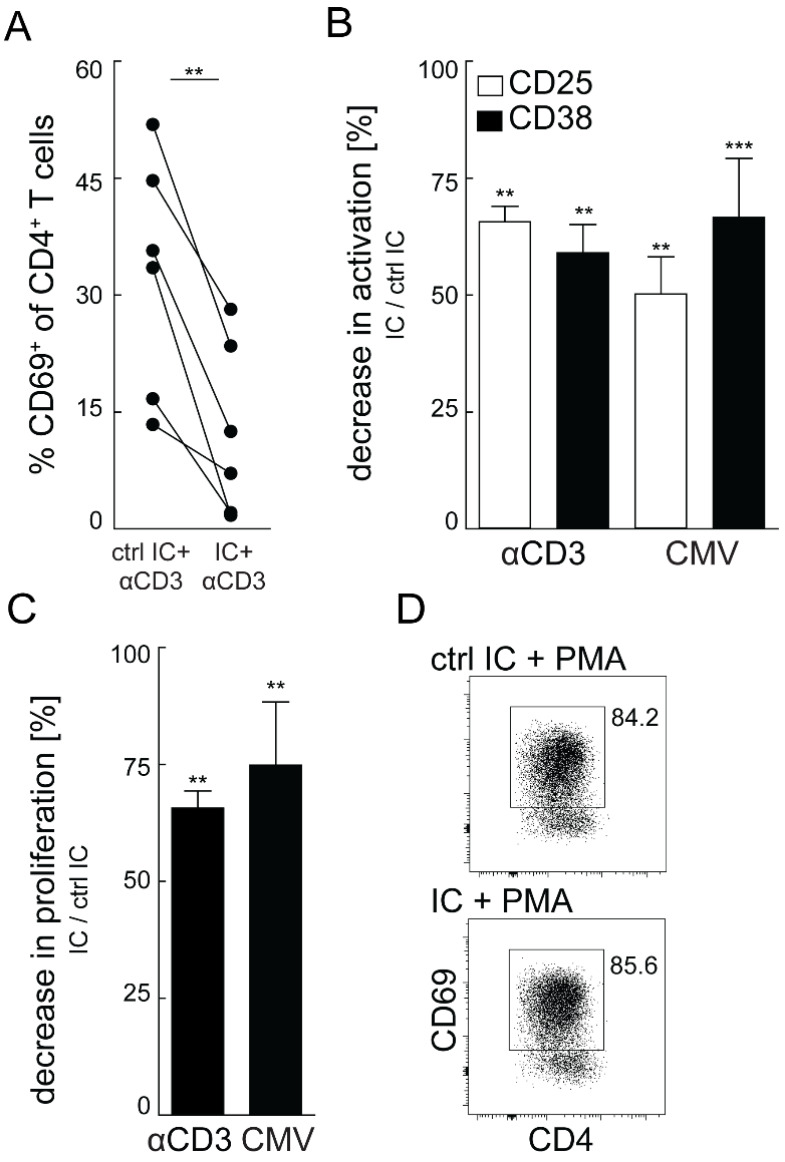

Figure 8: Gp120-anti-gp120 IC binding to the CD4 receptor impairs TCR-induced activation of CD4+ T cells.

CD4+ T cells were stimulated with ctrl IC or IC loaded autologous monocytes in the presence of anti-human CD3 mAb or monocytes that were previously loaded with overlapping peptides derived from immunodominant CMV protein pp65 and IE-1 as indicated (A-C). (A) Scatter plot shows CD4+ T cell activation measured by CD69 expression after 6 hours (n = 6, **p < 0.01, one-tailed, paired t-test). (B) Bar graph represents the relative decrease in activation induced by αCD3 (n = 3) or CMV peptides (n = 6) in terms of CD25 expression (white) and CD38 expression (black) in the presence of IC compared to ctrl IC (bars represent mean ± SEM, ***p < 0.001, **p < 0.01, one-tailed, paired t-test). (C) Bar graph represents decrease in activation induced by αCD3 (n=3) or CMV peptides (n = 6) in terms of proliferation measured by CFSE dilution in the presence of IC compared to ctrl IC (bars represent mean ± SEM, **p < 0.01, one-tailed, paired t-test). (D) Flow cytometry dot plot showing CD69 expression on CD4+ T cells stimulated for 6 hours with PMA+ionomycin in the presence of ctrl IC (top) or IC (bottom). n = nr of individual donors.

DISCUSSION

An outstanding peculiarity of HIV-1 is that its envelope surface glycoprotein has a very high affinity for the CD4 receptor (Myszka, Sweet et al. 2000), enabling HIV-1 and/or gp120 to directly interact and potentially interfere with CD4+ T cells. Such direct interference with the function of central regulators of the immune system is exceptional among human persistent viruses and represents a crucial factor in undermining HIV-specific and heterologous immune control. CD4 receptor binding by the HIV-1 envelope mediates not only viral infection (Dalgleish, Beverley et al. 1984, Sattentau 1988), but can also modify TCR-induced signaling by recruiting p56lck to the engaged TCRs (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991, Filipp, Leung et al. 2004, Kusumi, Ike et al. 2005), attributing the non-infectious interaction between gp120 and the CD4 receptor a potential role in modulating CD4+ T cell responses in a negative (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009) or a positive manner which is manifested by increased calcium signaling and IL-2R expression (Kornfeld, Cruikshank et al. 1988), transiently enhanced p56lck activity (Juszczak, Turchin et al. 1991, Hivroz, Mazerolles et al. 1993), activation of the transcription factors AP-1 (Chirmule, Goonewardena et al. 1995) and elevated proliferation (Oravecz and Norcross 1993). These studies convincingly demonstrate an important role of gp120 in modulating TCR-induced CD4+ T cell activation, however how gp120 diminishes or enhances the activation and whether it does so in an *in vivo* setting remains elusive. We therefore aimed in this study to re-investigate how gp120 modulates TCR-induced CD4+ T cell activation.

We confirm that a selective binding of gp120 to CD4 receptors on human CD4+ T cells differentially affects TCR-induced CD4+ T cell depending on the relative orientation of CD4 receptor versus TCR engagement. Simultaneous provision of immobilized agonistic anti-human CD3 mAb together with gp120 promotes full CD4+ T cell activation, indicating that concomitant crosslinking of CD4 receptors at the site of TCR engagement augments the proximal TCR signaling in a way that allows efficient signal propagation leading

to full CD4+ T cell activation. The amount of anti-CD3 mAb alone used in our experiments was not sufficient to overcome these signaling thresholds and consequently only supported moderate levels of CD4+ T cell activation. HIV-1 gp120's positive effect on low level CD4+ T cell activation was only observed if CD4 receptors and TCRs were simultaneously engaged in a spatially linked manner, indicating a synergistic mode of action of CD4 receptors and TCRs in inducing full CD4+ T cell activation. This likely reflects the physiological role of the CD4 receptor to recruit and activate p56lck at the site of TCR engagement upon interaction with MHC class II molecules on APCs and thereby significantly enhances TCR signaling efficiency in the presence of a weak TCR stimulus. In line with that, we show that also CD4 receptor cross-linking by plate immobilized anti-CD4 mAbs leads to comparably high levels of CD4+ T cell activation in the presence of low TCR stimulation as observed with plate-bound gp120.

Furthermore, our data demonstrate that gp120-containing ICs, similarly to gp120 alone, increase the CD4+ T cell response to anti-CD3 mAbs that were immobilized on the same surface. As opposed to this situation in which the TCR and CD4 receptor stimuli are both provided in an immobilized, plate bound manner, provision of the same stimuli in an APC bound fashion yields the opposite effect, namely reduced CD4+ T cell activation. We demonstrate that gp120 ICs which had been loaded on monocytes were rapidly and quantitatively transferred to CD4+ T cells and we suggest that this transfer is based on the different relative binding affinities of ICs to CD4+ T cells or monocytes. Because the binding of the gp120-anti-gp120 IC to the monocytes by means of IgG - Fc receptor (FcR) interaction is weak compared to the high affinity CD4-gp120 interaction (Myszka, Sweet et al. 2000), we speculate that gp120-anti-gp120 ICs are captured by CD4+ T cells from the monocytes in a stochastic process in which the ICs eventually localize on the cells to which they exhibit increased binding affinity. This transfer did not occur for the control IC in which the CD4BS on the gp120 is occupied and does consequently not allow any interaction with the CD4 receptor. Based on the fact that the transfer of the gp120-anti-gp120 ICs to CD4+ T cells was abrogated in the presence of a CD4 receptor blocking antibody, we exclude that the gp120-anti-gp120 ICs binds to the CD4+ T cells in an unspecific manner. Conversely to the role of plate immobilized gp120 in enhancing TCR-induced activation of CD4+ T cells, gp120 ICs adsorbed to CD4 receptors all around CD4+ T cells lead to substantial impairment of CD4+ T cell activation upon interaction with APCs which provide TCR engagement either by agonistic anti-human CD3 mAb or antigenic HLA class II/peptide complexes. CD4+ T cell activation

induced by PMA and ionomycin which bypasses early TCR-induced signaling is not affected by gp120 ICs and therefore suggests that gp120 ICs do not render CD4+ T cells completely unresponsive for any further activation but rather interfere with a proximal event of TCR signaling.

Furthermore, we addressed the question whether gp120-anti-gp120 ICs were generated upon the co-incubation of recombinant HIV-1 gp120 with two anti-gp120 mAbs with different specificities. We therefore evaluated the level of human IgG on the surface of CD4+ T cells that had been pre-incubated with gp120 complexed with either one or two anti-gp120 mAbs with different specificities. We detected increased levels of human IgG on the surface of CD4+ T cells that had been incubated with gp120 complexed with two different anti-gp120 mAbs compared to gp120 in combination with a single anti-gp120 mAb. This could indeed reflect the formation of ICs, which is only promoted in the presence of two antibodies with diverse specificities. However, we cannot exclude the possibility that such increased IgG levels could be attributed to the fact that gp120 allows twice as much anti-gp120 mAb binding if antibodies with two and not only single specificity are used. We therefore consider further assessment of IC formation by gel filtration which allows us to differentiate between antibodies attached to gp120 or IC formation according to the separation by size.

In conclusion, our data clearly demonstrate that gp120 is able to modulate the activation of CD4+ T cells by binding to the CD4 receptor and that the micro-anatomical environment of how gp120 cross-links CD4 receptors appears to be crucial for the ensuing effect on CD4+ T cell activation. Whereas plate-immobilized gp120 enhances low level of anti-CD3 mAb induced activation, gp120 adsorbed to CD4 receptors impairs high level of anti-CD3 mAb induced activation of CD4+ T cells. TCR stimulation was induced by an agonistic anti-CD3 mAb that binds to the CD3ε chain and consequently induces T cell activation irrespective of the antigenic specificity. In order to detect a positive effect on low level and a negative effect on high level TCR-induced CD4+ T cell activation by gp120 in its plate and cellular form, the appropriate level of activation was induced by diverse amounts of anti-CD3 mAb in these two different experimental set-ups. Of note, the exact same amount of anti-CD3 mAbs does not necessarily induce the same level of T cell activation in the plate and cellular assay, probably reflecting more and less efficient ways of TCR cross-linking. Our data offers an explanation for the long standing debate and contradictory results of previous *in vitro* studies, reporting

either enhanced or reduced CD4+ T cell activation upon gp120-CD4 receptor interaction. Whereas a stimulatory effect of gp120 or gp120 ICs is restricted to the "artificial" situation of immobilized stimuli, gp120 ICs might rather provoke the contrary under more physiological cell-based conditions what is more reflective of the *in vivo* situation in HIV-1 patients.

MATERIALS AND METHODS

Cell preparation

Citrate-phosphate-dextrose anti-coagulated buffy coats of healthy controls were purchased from the Swiss Red Cross (Blutspende Zurich, Switzerland) and EDTA anti-coagulated blood was obtained from HIV positive individuals from the University Hospital Zurich, Switzerland. Viral load in HIV-1 positive blood was determined at the Institute for Medical Virology at the University of Zurich (see Table 1 for detailed information). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphocyte Separation Media (LSM; PAA Laboratories, Pasching, Austria). Cells were washed in PBS and resuspended in RPM-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all reagents from PAA Laboratories, Pasching, Austria) what is referred to as RPMI-10 throughout the manuscript. If PBMCs were not directly used for experiments, they were cryopreserved in RPMI-1640 supplemented with 20% FBS and 10% DMSO (Sigma-Aldrich, Buchs, Switzerland). Cell viability was determined by 0.4% trypan blue exclusion (Invitrogen, Basel, Switzerland) and assessed to be greater than 90%. $CD4^+$ T cells and $CD14^+$ monocytes were isolated from PBMCs using anti-CD4 and -CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction.

CFSE labeling

For CFSE labeling, $CD4^+$ T cells were incubated at a concentration of 5×10^6 cells/ml in PBS supplemented with 10% FBS and 5 µM CFSE (Molecular Probes, Life Technologies Europe, Zug, Switzerland) for 11 minutes at 37° C. Afterwards, cells were washed twice with cold RPMI-10 to quench residual CFSE and finally resuspended with RPMI-10 at an appropriate cell concentration.

IC preparation

ICs were prepared by incubating mammalian cell-derived recombinant HIV-1 gp120 JR-FL (endotoxin level <1 EU/mg, Progenics Pharmaceuticals, Tarrytown, NY, USA) together with non-CD4BS anti-gp120 mAbs (clone 2G12 (Trkola, Purtscher et al. 1996) and clone 1-79 (Scheid, Mouquet et al. 2009)) or with CD4BS anti-gp120 mAbs (clone b6 (Pantophlet, Ollmann Saphire et al. 2003) and b12 (Barbas, Bjorling et al. 1992)) at a molar gp120:anti-gp120 mAb ratio of approximately 4:5 ($f.c._{gp120}$ 5 µg/ml) to prepare gp120-anti-gp120 IC and gp120-anti-gp120-CD4BS ctrl IC respectively. For the generation of gp120 containing ICs with

plasma from HIV-1+ donors, plasma from HIV-1- and HIV-1+ donors and recombinant HIV-1 gp120 JR-FL (endotoxin level <1 EU/mg, Progenics Pharmaceuticals, Tarrytown, NY, USA) were both diluted 1 to 100 and incubated for at least 2 hours.

In vitro CD4+ T cell stimulation assay with plate immobilized stimuli

2×10^6 CD4+ T cells/ml were activated with plate immobilized stimuli in a final volume of 100 µl RPMI-10. Plate immobilization of stimuli was done by incubating anti-human CD3 mAb (clone OKT3, eBioscience, Vienna, Austria), anti-human CD28 mAb (clone CD28.2 or clone CD28.6, eBioscience, Vienna, Austria), anti-human CD4 mAb (clone SK3, BD Biosciences, Allschwil, Switzerland), recombinant gp120 (Progenics Pharmaceuticals, Tarrytown, NY, USA) or ICs / ctrl ICs (prepared as described previously) in at total volume of 100 µl PBS on F-bottom 96 well plates (Nunc Maxisorp, Sigma Aldrich Chemie, Buchs, Switzerland) for at least 18 hours. 50 ng/well anti-human CD3 mAbs were used alone to induce sub-threshold T cell activation or in combination with 0.8 µg/well gp120, 1 µg/well anti-human CD4 mAb or 1 µg/well anti-human CD28 mAb. To induce full T cell activation by the TCR alone, 1 µg/well anti-human CD3 mAb were coated on each well. For the stimulation of mouse CD4+ T cells, anti-mouse CD3ε mAb and anti-mouse CD28 mAb (both from Biolegend, Lucerna Chem AG, Luzern, Switzerland) were used at similar concentrations as human specific antibodies. For internal assay controls, CD4+ T cells were left unstimulated or mitogenically stimulated with PMA (50 ng/ml, Sigma Aldrich Chemie, Buchs, Switzerland) in combination with ionomycin (500 ng/ml, Sigma Aldrich Chemie, Buchs, Switzerland). If indicated, the gp120 binding site on the CD4 receptor was blocked by pre-incubation of the CD4+ T cells with anti-human CD4 mAbs (10 µg/ml, clone SK3, eBioscience, Vienna, Austria) for 1 hour at 37° C. For intracellular analysis of IL-2 expression, brefeldin A (10 µg/ml, Sigma Aldrich Chemie, Buchs, Switzerland) was added during the final 4 hours of stimulation. Expression of CD40L, CD69 and IL-2 was analyzed after 6 hours and expression of CD25, CD38 as well as proliferation by CFSE dilution after 5 days of stimulation at 37° C as described under "Flow cytometric analysis".

In vitro CD4+ T cell stimulation assay with autologous monocytes

2×10^5 CD4+ T cells were stimulated with 1×10^5 autologous CD14+ monocytes in round-bottom 96 well cell culture plates in a final volume of 100 µl RPMI-10. Monocytes were loaded by sequential incubation of

anti-human CD3 mAb (0.5 to 10 ng/ml, clone OKT3, eBioscience, Vienna, Austria) for 30 minutes at 4° C or with CMV lysate (1:50, Virion, Rüschlikon, Switzerland) overnight at 37° C followed by incubation with ICs / ctrl ICs (f.c.$_{gp120}$ 5µg/ml) or gp120 containing ICs generated with plasma from HIV-1$^-$ and HIV-1$^+$ donors (final dilution factor 100 to 400) (as described under gp120 IC-anti-gp120 preparation) for 30 minutes at 4° C. Expression of CD69 was analyzed after 6 hours and expression of CD25 and CD38 as well as proliferation by CFSE dilution after 5 days of stimulation at 37° C as described under "Flow cytometric analysis".

Western blot analysis of (phospho)-LAT expression in CD4$^+$ T cells

For the analysis of levels of phosphorylated and unphosphorylated LAT, cells were cultured in RPM-1640 supplemented with 10% human serum (Type AB), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all reagents from PAA Laboratories, Pasching, Austria). CD4$^+$ T cells were pre-activated for 2 days with plate immobilized anti-human CD3 mAb (50 ng/well, eBioscience, Vienna, Austria) in combination with anti-human CD28 mAb (1 µg/well, eBioscience, Vienna, Austria), rested for 3 days in fresh medium and ultimately stimulated with plate immobilized stimuli as described under "*In vitro* CD4$^+$ T cell stimulation assay with plate immobilized stimuli". 6×10^5 cells were lysed in 30 µl cell lysis buffer (Cell Signaling Technologies, Bioconcept, Allschwil, Switzerland) in the presence of phosphatase inhibitor tablets (Thermo Fisher Scientific, Reinach, Switzerland). Proteins were separated on a 10% SDS polyacrylamide gel and transferred to 0.2 µm pore size nitrocellulose transfer membranes (Schleicher and Schuel, Dassel, Germany). The amount of phosphorylated LAT was determined by polyclonal anti-phospho-LAT antibody (Tyr171, 1:1000, Cell Signaling Technology, Bioconcept, Allschwil, Switzerland) followed by HRP-conjugated goat anti-rabbit antibody (1:5000, Jackson Immunoresearch, Suffolk, UK) using Amersham ECL technology (GE Healthcare, Glattbrugg, Switzerland). To measure the level of non-phosphorylated LAT protein, the membrane was reprobed after stripping with a polyclonal anti-LAT antibody (Cell Signaling Technology, Bioconcept, Allschwil, Switzerland). Signal intensities were quantified by ImageJ (National Institutes of Health). After background subtraction, the signal intensity of phosphorylated LAT was normalized to the amount of non-phosphorylated LAT and fold increase of normalized levels versus the unstimulated controls were calculated.

Flow cytometric analysis

Cells were surface stained with fluorochrome conjugated anti-CD3 (clone UHCT-1, BioLegend, Lucerna Chem AG, Luzern, Switzerland), anti-CD4 (clone SK3, BD Biosciences, Allschwil, Switzerland), anti-CD8 (clone SK1, BD Biosciences, Allschwil, Switzerland) and anti-CD14 (clone 61D3, eBioscience, Vienna, Austria), anti-CD25 (clone BC96, eBioscience, Vienna Austria), anti-CD38 (clone HIT-2a, BioLegend), anti-CD40L (clone TRAP1, BD Biosciences, Allschwil, Switzerland), anti-CD69 (clone FN50, BioLegend, Lucerna Chem AG, Luzern, Switzerland) or anti-human IgG (H+L) (Jackson Immunoresearch, Suffolk, UK) for 30 minutes at 4° C. Gp120 binding on $CD4^+$ T cells was assessed by Fluorescein isothiocyanate-(FITC, Sigma-Aldrich Chemie, Buchs, Switzerland) conjugated gp120 staining for 30 minutes at 4° C. For the analysis of intracellular IL-2 expression, surface staining was followed by a permeabilization step of 10 minutes at RT permeabilization (20% Lysing solution (BD Biosciences, Allschwil, Switzerland) and 0.05% Tween-20 (National Diagnostics, Chemie Brunschwig, Basel, Switzerland) and staining with anti-IL-2 (clone MQ1-17H12, BioLegend, Lucerna Chem AG, Luzern, Switzerland) for 30 minutes at RT. For compensation, a combination of anti-mouse Igκ/Negative Control Compensation Particles (BD Biosciences, Allschwil, Switzerland) or PBMCs were used. Cells were resuspended in PBS containing 1% PFA (Sigma Aldrich Chemie) before acquisition. Acquisition was done on a LSRII Flow Cytometer (BD Biosciences, Allschwil, Switzerland) using FACS DIVA software. Samples were acquired on the day of the analysis and usually 1-3×10^5 cells of interest were acquired. Doublets were excluded using side- and forward-scatter height and width parameters, negative biological or fluorescence minus one (FMO) controls were used to set gates. Data analysis was done with FlowJo software (TreeStar, San Carlos, CA, USA).

Fluorescent activated cell sorting (FACS)

PBMCs were surface stained using anti-CD3 (clone UHCT-1, BioLegend, Lucerna Chem AG, Luzern, Switzerland), anti-CD4 (clone RPA-T4, BD Biosciences, Allschwil, Switzerland), anti-CD45RA (clone HI100, eBioscience, Vienna, Austria) and anti-CD11a (cloneLFA-1, BD Biosciences, Allschwil, Switzerland) for 30 minutes at 4° C and cells were isolated on a FACS Aria (BD Biosciences, Allschwil, Switzerland) using FACS DIVA software.

Statistical analysis

Data was analyzed and plotted with GraphPad Prism (GraphPad, La Jolla, CA, USA) and results are illustrated as mean ± standard error of the mean (SEM). Statistical analysis was performed as specified in the figure legend. P-values less than 0.05 were considered statistically significant.

HIV-1 GP120 INTERFERES WITH THE FORMATION OF IMMUNOLOGICAL SYNAPSES AND THEREBY MODULATES CD4$^+$ T CELL ACTIVATION

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KZ and AO designed the experiments. KZ, AH and MR performed experiments and analyzed data. KZ and AO discussed data. AT contributed anti-gp120 antibodies. KZ and AO made the figures and wrote the manuscript.

SUMMARY

CD4 receptors play a crucial role in TCR-induced activation of $CD4^+$ T cells by recruiting and activating proximal TCR signaling kinases to the site of TCR engagement where the formation of immunological synapses (IS) is initiated. Sustained signaling induces a cytoskeleton-dependent accumulation of signaling units which goes along with the reorientation of the microtubule organizing centre (MTOC) toward the growing IS. CD4 receptor's ability to interact with TCR signaling molecules as well as with elements of the cytoskeleton offers non-infectious gp120 a way to interfere with $CD4^+$ T cell activation at the level of IS formation. Our previous findings attribute gp120 a role in modulating TCR-induced $CD4^+$ T cell activation in a positive or negative manner, depending on the relative orientation of CD4 receptor and TCR engagement. In this study, we demonstrate that the orientation of the MTOCs towards the site of TCR engagement occurred only if concomitant CD4 receptor cross-linking by gp120 was confined to the site of TCR triggering. In contrast, the orientation of the MTOC towards the site of TCR stimulation was abolished in $CD4^+$ T cells whose CD4 receptors had been cross-linked by gp120 ICs. Thus, we conclude that the manner of gp120/CD4 receptor cross-linking relative to the site of TCR engagement is crucial in the decision whether MTOCs can polarize to the site of engaged TCRs in a way that IS formation and hence $CD4^+$ T cell activation is supported.

INTRODUCTION

The fact that considerable amounts of soluble gp120 and gp160 are present in the blood (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Rychert, Strick et al. 2010) or lymphoid tissues of HIV$^+$ patients (Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009) which keeps a very high affinity to the CD4 receptor (Myszka, Sweet et al. 2000), prompted many researches to investigate how such non-infectious CD4-gp120 interaction affects the functionality of CD4$^+$ T cells in attempt to explain the characteristic decline of CD4$^+$ T cell function during HIV-1 infection in a quantitative and qualitative manner (Clerici, Stocks et al. 1989, Dolan, Clerici et al. 1995, Hazenberg, Hamann et al. 2000, McCune 2001, Grossman, Meier-Schellersheim et al. 2002, Guadalupe, Reay et al. 2003, Brenchley, Schacker et al. 2004, Mehandru, Poles et al. 2004, Gordon, Cervasi et al. 2010, Okoye and Picker 2013). Many studies revealed that non-infectious gp120 has a very prominent role in modulating the function of CD4$^+$ T cells (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Oravecz and Norcross 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009) what is based on the fact that CD4 receptors play a crucial role in potentiating TCR signaling efficiency (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991, Xiong, Kern et al. 2001, Filipp, Leung et al. 2004, Li, Dinner et al. 2004, Kusumi, Ike et al. 2005). Whereas the central tenet of these studies is beyond dispute, neither the functional outcome nor the underlying mechanism that explain how gp120 interferes with CD4$^+$ T cell activation is completely understood so far. Gp120-mediated interference with CD4$^+$ T cell activation was for instance attributed to its potential to induce CD4 receptor endocytosis (Chirmule, Kalyanaraman et al. 1988, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994), to block the MHCII-CD4 receptor interaction (Diamond, Sleckman et al. 1988, Rosenstein, Burakoff et al. 1990) or to modulate proximal TCR signaling (Mittler and Hoffmann 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Oyaizu, Chirmule et al. 1990, Goldman,

Jensen et al. 1994) by influencing the activation and recruitment of the p56lck to the site of TCR engagement (Goldman, Crabtree et al. 1997, Nyakeriga, Fichtenbaum et al. 2009).

Our data confirmed that gp120 has the potential to enhance or diminish TCR-induced $CD4^+$ T cell activation and we provide evidence that these opposing effects are attributed to the relative orientation of CD4 receptor and TCR engagement. Concomitant gp120-mediated CD4 receptor cross-linking at the site of TCR engagement by low amounts of plate bound agonistic anti-CD3 mAbs lead to high $CD4^+$ T cell activation whereas the low amount of agonistic anti-CD3 mAbs was not sufficient to exceed signaling thresholds for efficient TCR signaling propagation required to induce $CD4^+$ T cell activation. In contrast, in an assay in which $CD4^+$ T cells are exposed to anti-CD3 mAb- / IC -loaded monocytes, the efficient transfer of gp120 ICs from monocytes interacting $CD4^+$ T cells what substantially impaired $CD4^+$ T cell activation induced upon TCR stimulation.

TCR triggering beyond a certain signaling threshold induces the clustering of TCR signaling molecules to the site of initial TCR engagement in a cytoskeleton dependent manner (Monks, Freiberg et al. 1998, Saito and Yokosuka 2006, Seminario and Bunnell 2008, Dustin and Groves 2012) which upon sustained signaling leads to the formation of immunological synapses (IS) (Wulfing and Davis 1998, Dustin and Cooper 2000, Sancho, Vicente-Manzanares et al. 2002, Billadeau, Nolz et al. 2007, Martin-Cofreces, Alarcon et al. 2011). Since CD4 receptors are physically associated with protein tyrosine kinase p56lck (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991) and elements of the cytoskeleton (Geppert and Lipsky 1991, Rozdzial, Malissen et al. 1995, Zeyda and Stulnig 2006), their engagement allows to modulate the IS formation at the level of proximal TCR signaling as well as at the level of cytoskeleton dependent dynamics. Importantly, the formation of IS is accompanied by a repositioning of the MTOC towards the growing synapse (Kupfer, Swain et al. 1987, Martin-Cofreces, Robles-Valero et al. 2008).

We demonstrate that gp120 cross-linking of CD4 receptors indeed influenced $CD4^+$ T cell activation by interfering with the formation of IS. Simultaneous CD4 receptor cross-linking at the site of the growing synapse beyond the engaged TCRs, induced a preferential orientation of the MTOC towards the site of TCR engagement which is characteristic for IS formation and required for $CD4^+$ T cell activation. However, IS

formation at the site of TCR engagement seems to be hindered in $CD4^+$ T cells whose CD4 receptors are cross-linked by gp120-anti-gp120 ICs what was reflected by MTOCs that are not preferentially oriented towards the site of TCR engagement. All in all, our data suggest the gp120 has the potential to interfere with the activation of $CD4^+$ T cells by interfering with the process of IS formation which is an indispensable prerequisite for efficient TCR signal propagation and hence $CD4^+$ T cell activation.

RESULTS

Gp120-anti-gp120 ICs do not induce CD4 receptor mediated endocytosis in CD4⁺ T cells

Gp120 binding to CD4 receptors was suggested to induce the endocytosis of CD4 receptors and thus impair CD4⁺ T cell activation (Chirmule, Kalyanaraman et al. 1988, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994). It is therefore conceivable that the transfer of gp120-anti-gp120 ICs from monocytes to CD4 receptors on CD4⁺ T cells is followed by endocytosis of CD4 receptors what would explain the impaired TCR-responsiveness of gp120-anti-gp120 IC coated CD4⁺ T cells in our cell-based *in vitro* stimulation assay. Such a mechanism, however, would not affect CD4⁺ T cell activation induced by the irreversible immobilization of the stimuli on a planar substrate. ICs on the surface of CD4⁺ T cells proved to stay relatively constant, thereby excluding CD4 receptor endocytosis as major factor in impairing CD4⁺ T cell activation in our assay (Figure 1).

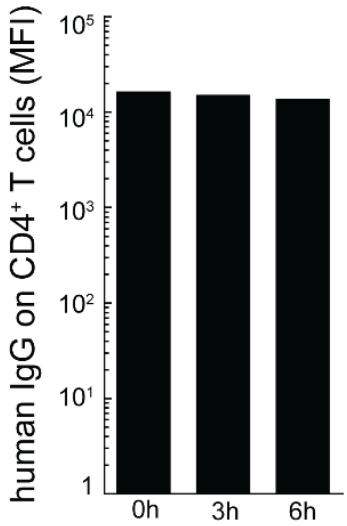

Figure 1: Gp120-anti-gp120 IC binding to CD4 receptors does not lead to endocytosis associated loss of human IgG signal on the CD4⁺ T cell surface within 6 hours.

CD4⁺ T cells from healthy donors were incubated with gp120-anti-gp120 ICs and the presence of human IgG on the surface of CD4⁺ T cells was analyzed after 0, 3 or 6 hours by flow cytometry. Bars of one representative experiment depict MFI of human IgG signal.

Monocytes do not produce IL-15 upon FcR engagement by ICs

Our *in vitro* experiments depict an efficient transfer of gp120-anti-gp120 ICs from monocytes - on which ICs are relatively loosely attached - to CD4⁺ T cells whose CD4 receptors bind gp120 with very high affinity (Myszka, Sweet et al. 2000). In contrast, ctrl ICs remain on the monocytes upon interaction with CD4⁺ T cells due to the absence of free a CD4BS on the gp120 within these ICs. It is therefore possible that FcR engagement on monocytes by ctrl ICs induces their activation (Neely, Robbins et al. 2001) which in turn might enhance CD4⁺ T cell activation by a soluble mediator such as IL-15, particularly in the presence of TCR engagement (Bastidas, unpublished observation). The production of IL-15 in monocytes, however, was

not induced upon FcR engagement by ICs what makes it unlikely that a monocyte-derived co-stimulatory signal would substantially influence levels of TCR-induced CD4+ T cell activation in our experiments.

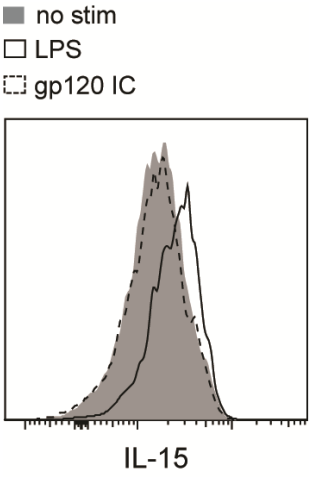

Figure 2: The expression of IL-15 is not increased upon incubation of monocytes with gp120 ICs within 6 hours.

CD14+ monocytes were stimulated with LPS (1 ng/ml) or gp120-anti-gp120 ICs that had been generated at a molar gp120:anti-gp120 mAb ratio of approximately 4:5 ($f.c._{gp120}$ 5 µg/ml) and the expression of IL-15 was analyzed after 6 hours. Flow cytometry histogram depicts IL-15 expression on the surface of CD14+ monocytes.

CD4 receptor cross-linking by gp120-anti-gp120 IC interferes with the clustering of signaling complexes at the site of TCR engagement

Based on the previous findings that suggest that gp120 binding to the CD4 receptor has the potential to interfere with the recruitment of the p56lck to the site of TCR engagement and growing IS (Goldman, Crabtree et al. 1997, Nyakeriga, Fichtenbaum et al. 2009), we were wondering whether gp120 interferes with CD4+ T cell activation at the level of IS formation. TCR triggering induces a clustering of signaling complexes beyond the engaged TCRs which upon sustained signaling gives rise to the formation of IS (Monks, Freiberg et al. 1998, Saito and Yokosuka 2006, Seminario and Bunnell 2008, Dustin and Groves 2012). Such accumulation of surface and signaling molecules depends on dynamic reorganization of the actin and tubulin-based T cell cytoskeleton (Wulfing and Davis 1998, Dustin and Cooper 2000, Sancho, Vicente-Manzanares et al. 2002, Billadeau, Nolz et al. 2007, Martin-Cofreces, Alarcon et al. 2011) which is also reflected by the repositioning of the MTOC towards the site of stimulation (Kupfer, Swain et al. 1987, Martin-Cofreces, Robles-Valero et al. 2008). CD4 receptors are essential in proximal TCR signaling (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991) by recruiting p56lck to the establishing IS (Filipp, Leung et al. 2004, Kusumi, Ike et al. 2005). Since many TCR signaling molecules (e.g. the CD4 receptor) are indirectly or directly linked to the cytoskeleton (Geppert and Lipsky 1991, Rozdzial, Malissen et al. 1995, Zeyda and Stulnig 2006), this opens the possibility that gp120 binding to the CD4 receptor might interfere with IS formation. Accordingly, when

cytoskeleton dynamics was abrogated by disrupting actin polymerization by Cytochalasin D (CycD) (Valitutti, Dessing et al. 1995), TCR signaling induced by concomitant CD4/TCR cross-linking at a defined site did not promote full $CD4^+$ T cell activation, whereas a functional, dynamic cytoskeleton supported the accumulation of signaling complexes underneath the engaged TCRs such that local signaling strength resulted in full $CD4^+$ T cell activation (Figure 3A). Importantly, TCR and CD4-independent stimulation by PMA and ionomycin was not dependent on a functional, dynamic actin cytoskeleton (Figure 3A).

We speculate that spatially and temporally linked engagement of TCR and CD4 (as in the case of plate-immobilized anti-human CD3 mAb and gp120) would favor IS formation. However if CD4 receptors are cross-linked over the entire surface of the $CD4^+$ T cell upon capturing ICs from monocytes, we suggest that the cytoskeletal dynamics which is required for IS formation might be impeded in such IC decorated $CD4^+$ T cells. To test this hypothesis, we investigated MTOC positioning towards the growing synapse which is a marker for IS formation (Kupfer, Swain et al. 1987, Martin-Cofreces, Robles-Valero et al. 2008) by staining for β-tubulin. Figure 3B shows representative confocal z-stack images of $CD4^+$ T cells stimulated by plate-immobilized anti-human CD3 mAb and gp120. The left lane shows a $CD4^+$ T cell in which the MTOC is oriented towards the stimuli (referred to as polarized), whereas the right lane depicts a $CD4^+$ T cell in which the MTOC is not polarized. Figure 3E shows representative immunofluorescence images of $CD4^+$ T cells which were stimulated by anti-human CD3 mAb loaded monocytes. On the top, a situation is shown in which the MTOCs of both APC engaging $CD4^+$ T cells point towards the site of cell-cell contact (filled arrow, polarized), whereas the MTOC of the APC-engaging $CD4^+$ T cell shown in the bottom image points away from the cell-cell contact (open arrow, not polarized). Going in line with the increased $CD4^+$ T cell activation induced by plate immobilized anti-human CD3 mAb and gp120 or IC, MTOC polarization towards the site of TCR stimulation was increased in the presence of IC (Figure 3C bottom and Figure 3D) in contrast to the ctrl IC (Figure 3C top and Figure 3D). In striking contrast, reduced TCR-induced $CD4^+$ T cell activation in the presence of IC on monocytes was associated with impaired MTOC polarization towards the $CD4^+$ T cell-APC interface, whereas the presence of the ctrl IC allowed efficient polarization of MTOCs towards the site of TCR engagement (Figure 3F and 3G).

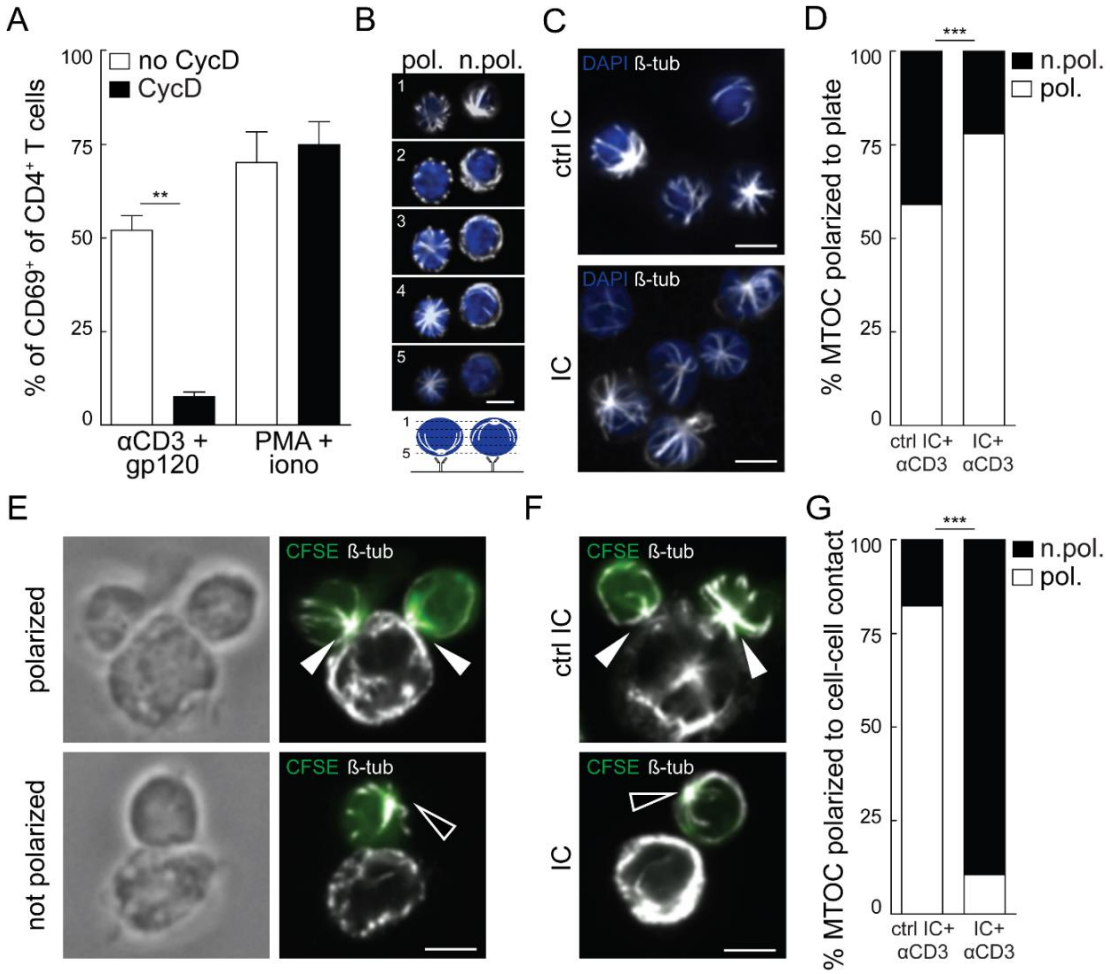

Figure 3: Gp120-anti-gp120 ICs interfere with the orientation of the MTOCs towards the site of TCR engagement which is an essential for CD4⁺ T cell activation.

(A) Bar graphs depict the expression of CD69 on CD4⁺ T cells stimulated for 6 hours with plate immobilized αCD3+gp120 or PMA+ionomycin in the presence (black bars) or absence (white bars) of Cytochalasin D (n = 3) (n= nr of individual donors, bars represent mean ± SEM, **p<0.01, one-tailed, paired t-test). CD4⁺ T cells were stimulated for 30 minutes with anti-human CD3 mAb in the presence of ctrl IC or IC immobilized on coverslips showing nuclei by DAPI staining (blue) and MTOC by β-tubulin staining (white) (B-D). (B) Exemplary z-stacks of immunofluorescence images depicting two CD4⁺ T cells with differential MTOC polarization (left, polarized and right, not polarized) whereas MTOC polarization is referred to as MTOC positioning towards the site of TCR engagement. (C) Representative immunofluorescence images of CD4⁺ T cells in z-stack close to the coverslip in the presence of ctrl IC (top) or IC (bottom). (D) Bar graph summarizing MTOC polarization to the stimuli (in white) and random polarization (in black) in the presence of ctrl IC (n = 166) and IC (n = 180) (n = number of individual cells, ***p <0.001, chi-square approximation). (E-G) CD4⁺ T cells were stimulated for 2 hours with monocytes loaded with anti-human CD3 mAb in the presence of ctrl IC or IC showing nuclei by DAPI staining (blue), MTOC by β-tubulin staining (white) and CFSE-labeled CD4⁺ T cells (green). (E) Exemplary immunofluorescence images showing MTOCs which are polarized (filled arrow) or not polarized (empty arrow) to the site of cell-cell contact or not. (F) Representative immunofluorescence images of CD4⁺ T cells in the presence of ctrl IC (top) or IC (bottom). (G) Bar graph summarizing MTOC polarization towards the site of cell-cell contact (white) and random polarization (black) of CD4⁺ T cells in the presence of ctrl IC (n = 15) or IC (n = 14) loaded monocytes (n = number of individual cell-cell interactions, ***p < 0.001, Fisher's exact test), scale bar = 5µm.

DISCUSSION

HIV-1's ability to interfere with the central regulators of the immune system by means of the high affinity interaction between gp120 and the CD4 receptors (Myszka, Sweet et al. 2000) are suggested to contribute to the progressive immunodeficiency during HIV-1 infection. Whereas there is general agreement that gp120-CD4 receptor interaction indeed interferes with TCR-induced $CD4^+$ T cell activation based on various *in vitro* experiments (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Oravecz and Norcross 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009), neither the exact functional consequences nor the underlying mechanism are completely understood so far. Our previous findings suggest that enhanced or diminished levels of TCR-induced $CD4^+$ T cell activation can be ascribed to the relative orientation of CD4 receptor and TCR engagement which influences the efficiency of TCR signaling. We exclude the possibility that binding of ICs to CD4 receptors induces the endocytosis of CD4 receptors which had been described as mechanism for the gp120-mediated reduction of $CD4^+$ T cell activation (Chirmule, Kalyanaraman et al. 1988, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994). Furthermore, our data cannot confirm that gp120 prevents $CD4^+$ T cell activation by interfering with MHC II-CD4 receptor interaction which is required for optimal $CD4^+$ T cell activation (Diamond, Sleckman et al. 1988, Rosenstein, Burakoff et al. 1990), since we observed the inhibitory effect of gp120 on $CD4^+$ T cells activated upon MHC II independent TCR engagement by anti-human CD3 mAbs. Moreover, we exclude that ICs manifest reduced $CD4^+$ T cell activation due to TCRs being sterically hindered from contacting their activating ligands on the APC, since both the ctrl IC and IC are similarly localized at the site of cell-cell contact and only the latter ones affect $CD4^+$ T cell activation. On top of that, a monocyte-derived activating signal (e.g. IL-15) seems to be negligible in affecting the level of $CD4^+$ T cell activation in our experimental set-up.

We suggest that the basis for gp120-mediated interference with TCR-induced $CD4^+$ T cell activation relies on the fact that CD4 receptors not only interact with the TCR-CD3 complex and p56lck but also with

elements of the cytoskeleton (Geppert and Lipsky 1991, Rozdzial, Malissen et al. 1995, Zeyda and Stulnig 2006), and consequently substantially interfere with the cytoskeletal dependent rearrangement of signaling molecules towards the site of TCR stimulation which ultimately regulates $CD4^+$ T cell activation (Wulfing and Davis 1998, Dustin and Cooper 2000, Sancho, Vicente-Manzanares et al. 2002, Billadeau, Nolz et al. 2007, Martin-Cofreces, Alarcon et al. 2011). This reasoning is supported by the observation that cross-linking of TCR/CD4 receptors in close proximity at a confined site only supports $CD4^+$ T cell activation if the cytoskeleton is highly dynamic but not if the actin polymerization is blocked by Cytochalasin D. MTOC orientation towards the site of TCR engagement is one hallmark of a functional IS (Kupfer and Dennert 1984, Martin-Cofreces, Robles-Valero et al. 2008) and our results clearly demonstrate that MTOC positioning is preferentially oriented towards plate co-immobilized anti-human CD3 mAb and gp120. This indicates that cross-linking of CD4/TCR in close proximity allows cytoskeleton-directed accumulation of signaling molecules towards the site of engagement, thereby supporting full $CD4^+$ T cell activation. In contrast, IC binding to CD4 receptors of $CD4^+$ T cells likely restricts cytoskeletal rearrangement by impairing lateral mobility of CD4 receptors and their cytoskeleton associated framework. In line with that we demonstrate that cross-linking of CD4 receptors by ICs not just at the site of TCR engagement hinders the accumulation of signaling units beyond the engaged TCRs, reflected by impaired MTOC orientation towards the site of TCR stimulation and hence compromised T cell activation.

All in all, these results attribute gp120 a prominent role in interfering with $CD4^+$ T cell activation at the level of IS formation. This goes in line with previous reports disclosing a role of gp120 in preventing proximal TCR signaling (Mittler and Hoffmann 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Oyaizu, Chirmule et al. 1990, Goldman, Jensen et al. 1994, Waterman, Marschner et al. 2012), being possibly a direct consequence of impaired IS formation. On top of that, supportive evidence comes from a study which describes that CD4 receptor engagement impairs the recruitment of p56lck to the growing IS what consequently prevents efficient $CD4^+$ T cell activation (Nyakeriga, Fichtenbaum et al. 2009).

MATERIALS AND METHODS

Cell preparation

Citrate-phosphate-dextrose anti-coagulated buffy coats of healthy controls were purchased from the Swiss Red Cross (Blutspende Zurich, Switzerland) and EDTA anti-coagulated blood was obtained from HIV positive individuals from the University Hospital Zurich, Switzerland. Viral load in HIV-1 positive blood was determined at the Institute for Medical Virology at the University of Zurich (see Table 1 for detailed information). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphocyte Separation Media (LSM; PAA Laboratories, Pasching, Austria). Cells were washed in PBS and resuspended in RPM-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all reagents from PAA Laboratories, Pasching, Austria) what is referred to as RPMI-10 throughout the manuscript. If PBMCs were not directly used for experiments, they were cryopreserved in RPMI-1640 supplemented with 20% FBS and 10% DMSO (Sigma-Aldrich, Buchs, Switzerland). Cell viability was determined by 0.4% trypan blue exclusion (Invitrogen, Basel, Switzerland) and assessed to be greater than 90%. $CD4^+$ T cells and $CD14^+$ monocytes were isolated from PBMCs using anti-CD4 and -CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction.

IC preparation

ICs were prepared by incubating mammalian cell-derived recombinant HIV-1 gp120 JR-FL (endotoxin level <1 EU/mg, Progenics Pharmaceuticals, Tarrytown, NY, USA) together with non-CD4BS anti-gp120 mAbs (clone 2G12 (Trkola, Purtscher et al. 1996) and clone 1-79 (Scheid, Mouquet et al. 2009)) or with CD4BS anti-gp120 mAbs (clone b6 (Pantophlet, Ollmann Saphire et al. 2003) and b12 (Barbas, Bjorling et al. 1992)) at a molar gp120:anti-gp120 mAb ratio of approximately 4:5 ($f.c._{gp120}$ 5 µg/ml) to prepare gp120-anti-gp120 IC and gp120-anti-gp120-CD4BS ctrl IC respectively. For the generation of gp120 containing ICs with plasma from $HIV\text{-}1^+$ donors, plasma from $HIV\text{-}1^-$ and $HIV\text{-}1^+$ donors and recombinant HIV-1 gp120 JR-FL (endotoxin level <1 EU/mg, Progenics Pharmaceuticals, Tarrytown, NY, USA) were both diluted 1 to 100 and incubated for at least 2 hours.

Analysis of IC on the surface of $CD4^+$ T cells

$CD4^+$ T cells were incubated with IC prepared at a molar gp120:anti-gp120 mAb ratio of approximately 4:5 ($f.c._{gp120}$ 5 µg/ml) for 3 or 6 hours at 37° C and the amount of IC on the surface was investigated by flow cytometry staining for anti-human IgG.

Analysis of IL-15 expression on $CD14^+$ monocytes

$CD14^+$ monocytes were stimulated with LPS (1 ng/ml) or gp120-anti-gp120 ICs that had been generated at a molar gp120:anti-gp120 mAb ratio of approximately 4:5 ($f.c._{gp120}$ 5 µg/ml) and the surface expression of IL-15 was analyzed after 3 and 6 hours by flow cytometric analysis.

In vitro $CD4^+$ T cell stimulation assay with autologous monocytes

2×10^5 $CD4^+$ T cells were stimulated with 1×10^5 autologous $CD14^+$ monocytes in round-bottom 96 well cell culture plates in a final volume of 100 µl RPMI-10. Monocytes were loaded by sequential incubation of anti-human CD3 mAb (0.5 to 10 ng/ml, clone OKT3, eBioscience, Vienna, Austria) for 30 minutes at 4° C or with CMV lysate (1:50, Virion, Rüschlikon, Switzerland) overnight at 37° C followed by incubation with ICs / ctrl ICs ($f.c._{gp120}$ 5µg/ml) or gp120 containing ICs generated with plasma from $HIV\text{-}1^-$ and $HIV\text{-}1^+$ donors (final dilution factor 100 to 400) (as described under gp120 IC-anti-gp120 preparation) for 30 minutes at 4° C. Expression of CD69 was analyzed after 6 hours and expression of CD25 and CD38 as well as proliferation by CFSE dilution after 5 days of stimulation at 37° C as described under "Flow cytometric analysis".

MTOC polarization analysis of $CD4^+$ T cells in the context of plate (i) and cellular (ii) stimulation by immunofluorescence confocal microscopy

(i) For analysis of MTOC polarization in the context of plate stimulation, $CD4^+$ T cells were incubated for 2 hours on 12 mm round coverslips (Karl Hecht Assistant, Altnau, Switzerland) that have previously been coated for at least 18 hours with anti-human CD3 mAb (50 ng/ml) in combination with ICs / ctrl ICs ($f.c._{gp120}$ 5 µg/ml) diluted in PBS containing 0.0025% poly-L-lysine (Sigma Aldrich Chemie, Buchs, Switzerland).

(ii) MTOC polarization analysis in the context of cellular stimulation was performed as described under "*In vitro* $CD4^+$ T cell stimulation assay with autologous monocytes" except that

stimulation was performed for 2 hours only and cells were adhered onto poly-L-lysine (0.0025 %, Sigma Aldrich Chemie, Buchs, Switzerland) coated coverslips.

(iii) (i and ii) After stimulation, cells were fixed with PBS containing 4% PFA (Sigma Aldrich Chemie, Buchs, Switzerland) for 15 minutes at RT, followed by permeabilization (20% Lysing solution (BD Biosciences, Allschwil, Switzerland) and 0.05% Tween-20 (National Diagnostics, Chemie Brunschwig, Basel, Switzerland)) for 10 minutes at RT. MTOC structures were visualized by staining with an anti-tubulin-β mAb (clone 9F3, Cell Signaling Technology, Bioconcept, Allschwil, Switzerland) overnight at 4° C in a humid chamber and Cy3-conjugated anti-rabbit ab (Jackson Immunoresearch, Suffolk, UK) for 30 minutes at RT.

Immunofluorescence confocal microscopy

After staining, cells were fixed with PBS containing 4% PFA (Sigma Aldrich Chemie, Buchs, Switzerland) for 15 minutes at RT and briefly rinsed with destilled H_2O before mounting in VectaShield (Vector Laboratories, Burlingame, CA, USA) containing 0.1% DAPI (Sigma Aldrich Chemie) for visualization of nuclear DNA. Confocal immunofluorescence microscopy was performed with an inverted confocal microscope (Axiovert 200, Carl Zeiss) equipped with an oil-phase contrast objective (63× oil objective, Plan Neofluar, 1.25 numerical aperature, Carl Zeiss) and a CSU-X1 spinning-disk confocal unit (Yokogawa) and a solid state laser unit with four laser lines (405, 488, 561, 647, Toptica). Data analysis was done with Volocity 5.0.3 (Improvision, Coventry, UK). Quantification of IC staining was done by evaluation of the integrated signal density by ImageJ (National Institutes of Health, USA), values were normalized to cell size. Analysis of MTOC polarization was done by visual scoring as described in Figure 3B and 3E.

Flow cytometric analysis

Cells were surface stained with fluorochrome conjugated anti-CD3 (clone UHCT-1, BioLegend, Lucerna Chem AG, Luzern, Switzerland), anti-CD4 (clone SK3, BD Biosciences, Allschwil, Switzerland) and anti-CD14 (clone 61D3, eBioscience, Vienna, Austria), anti-IL-15 (Clone 34559, R&D Systems, Minneapolis, USA) or anti-human IgG (H+L) (Jackson Immunoresearch, Suffolk, UK) for 30 minutes at 4° C. For compensation, a combination of anti-mouse Igκ/Negative Control Compensation Particles (BD Biosciences, Allschwil, Switzerland) or PBMCs were used. Cells were resuspended in PBS containing 1%

PFA (Sigma Aldrich Chemie) before acquisition. Acquisition was done on a LSRII Flow Cytometer (BD Biosciences, Allschwil, Switzerland) using FACS DIVA software. Samples were acquired on the day of the analysis and usually 1-3 $\times 10^5$ cells of interest were acquired. Doublets were excluded using side- and forward-scatter height and width parameters, negative biological or fluorescence minus one (FMO) controls were used to set gates. Data analysis was done with FlowJo software (TreeStar, San Carlos, CA, USA).

Statistical analysis

Data was analyzed and plotted with GraphPad Prism (GraphPad, La Jolla, CA, USA) and results are illustrated as mean ± standard error of the mean (SEM). Statistical analysis was performed as specified in the figure legend. P-values less than 0.05 were considered statistically significant.

CONTRIBUTION OF GP120-ANTI-GP120 ICS TO CD4$^+$ T CELL FUNCTION *IN VIVO*

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KZ and AO designed the experiments. KZ and TL performed experiments and analyzed data. KZ and AO discussed data. AT, TL, contributed plasma samples and anti-gp120 antibodies. HG enrolled patients. KZ and AO made the figures and wrote the manuscript.

SUMMARY

Since the HIV-1 envelope glycoprotein is readily shed from the virus or from infected cells on the one side and elicits a strong antibody response on the other side, gp120 containing ICs are abundantly present in HIV+ donors. In line with that, we showed previously that ICs that had been generated with recombinant gp120 and anti-gp120 mAbs are efficiently adsorbed to $CD4^+$ T cells where they substantially impair the responsiveness to TCR stimuli *in vitro*. In this study, we provide evidence that gp120 might contribute to the progressive decline of the $CD4^+$ T cell function *in vivo* by demonstrating that $CD4^+$ T cells in the blood from HIV-1 patients are covered with ICs which at least partially contain gp120. Furthermore, we demonstrate that polyclonal anti-gp120 antibodies in HIV-1 patients have the capacity to from gp120 containing ICs which bind efficiently to the surface of $CD4^+$ T cells and thereby impair TCR-induced $CD4^+$ T cell activation.

INTRODUCTION

Loss of $CD4^+$ T cell function (Hazenberg, Hamann et al. 2000, McCune 2001, Grossman, Meier-Schellersheim et al. 2002, Okoye and Picker 2013) is a central pathogenic feature of the progressive HIV-1 associated immunodeficiency (Douek, Roederer et al. 2009). Since the levels of infectious virus and productively infected cells are low (Harper, Marselle et al. 1986, Douek, Picker et al. 2003), mechanisms apart from direct HIV-1 cytopathicity (Lenardo, Angleman et al. 2002) must account for the extensive $CD4^+$ T cell dysfunction during HIV-1 infection. The fact that not only HIV-1 but also soluble gp120 binds to CD4 receptors with very high affinity (Myszka, Sweet et al. 2000) attributes non-infectious gp120-CD4 receptor interaction a prominent role in provoking $CD4^+$ T cell dysfunction, however it's *in vivo* relevance is poorly investigated so far.

HIV-1 gp120 and gp160 are present in the plasma (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009, Rychert, Strick et al. 2010) due to shedding of the envelope from HIV-1 virions or from infected cells (Gelderblom, Reupke et al. 1985, Schneider, Kaaden et al. 1986, Pyle, Bess et al. 1987). Since the HIV-1 envelope elicits a strong antibody response (Lyerly, Reed et al. 1987, Doria-Rose, Klein et al. 2009, McCoy and Weiss 2013), gp120-containing ICs are readily formed and indeed abundantly present *in vivo* (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1996, Daniel, Susal et al. 1998, Suzuki, Gatanaga et al. 2014). Based on the documented presence of these ICs *in vivo*, we generated gp120 containing ICs by co-incubation of recombinant gp120 and anti-gp120 mAbs and found that the exposure of $CD4^+$ T cells to monocytes that had been loaded with these ICs lead to an efficient transfer of gp120-anti-gp120 ICs to the CD4 receptors. Furthermore, we showed that gp120-anti-gp120 IC binding to CD4 receptors renders $CD4^+$ T cells hyporesponsive to subsequent TCR stimulation which goes in line with a plethora of previous *in vitro* studies that attribute gp120 an inhibitory role of $CD4^+$ T cell function (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009).

Based on our *in vitro* observation that gp120-anti-g120 ICs are readily transferred from monocytes to $CD4^+$ T cells, it is conceivable that gp120-containing ICs - either in a soluble form or trapped by host cells via Fc receptors - are readily adsorbed to CD4 receptors and could supposedly be found on the surface of $CD4^+$ T cells in HIV-1 patients. Consistent with this notion, we demonstrate in this study that $CD4^+$ T cells isolated from the blood of HIV-1 patients are indeed covered with ICs. Preliminary data suggest that these ICs contain gp120 what corroborates their potential in contributing to the $CD4^+$ T cell dysfunction *in vivo*. Furthermore, we demonstrate that the repertoire of *in vivo* circulating polyclonal antibodies directed against the gp120 of HIV-1 allows the formation of gp120-containing ICs in the presence of soluble gp120 which have the potential to be adsorbed by $CD4^+$ T cells on which they provoke $CD4^+$ T cell unresponsiveness.

RESULTS

CD4⁺ T cells from HIV-1 patients are covered with (gp120 containing) ICs

Based on the fact that gp120 is present in plasma from HIV-1 patients (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Rychert, Strick et al. 2010) and that HIV-1 infection is associated with robust antibody titers specific for HIV-1 surface structures (Lyerly, Reed et al. 1987, Nara, Garrity et al. 1991, Doria-Rose, Klein et al. 2009, McCoy and Weiss 2013), gp120-anti-gp120 ICs are suggested to be the predominant form of gp120 *in vivo* (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1996). Such complexes might either circulate, or more likely be bound by Fc-receptor or complement-receptor expressing host cells. Our *in vitro* experiments demonstrate that such gp120-anti-gp120 ICs were efficiently transferred from monocytes to CD4⁺ T cells, suggesting that CD4⁺ T cells from HIV-1 patients might be decorated with gp120 containing ICs upon capturing them in their soluble or cell-bound form. In line with that, we found that CD4⁺ T cells isolated from blood of HIV-1 patients exhibited significantly elevated anti-human IgG staining compared to CD4⁺ T cells isolated from blood of healthy controls (Figure 1A and 1B), which is indicative for the presence of ICs on their surface. Furthermore, staining with two human anti-gp120 mAbs in combination with an anti-human IgG antibody disclosed increased IgG levels on CD4⁺ T cells from HIV+ donors compared to IgG levels without the addition of the two human anti-gp120 mAbs (Figure 1C), suggesting that human IgG antibodies on the surface of CD4⁺ T cells from HIV+ donors might at least partially be attributed to the presence of gp120 in these ICs.

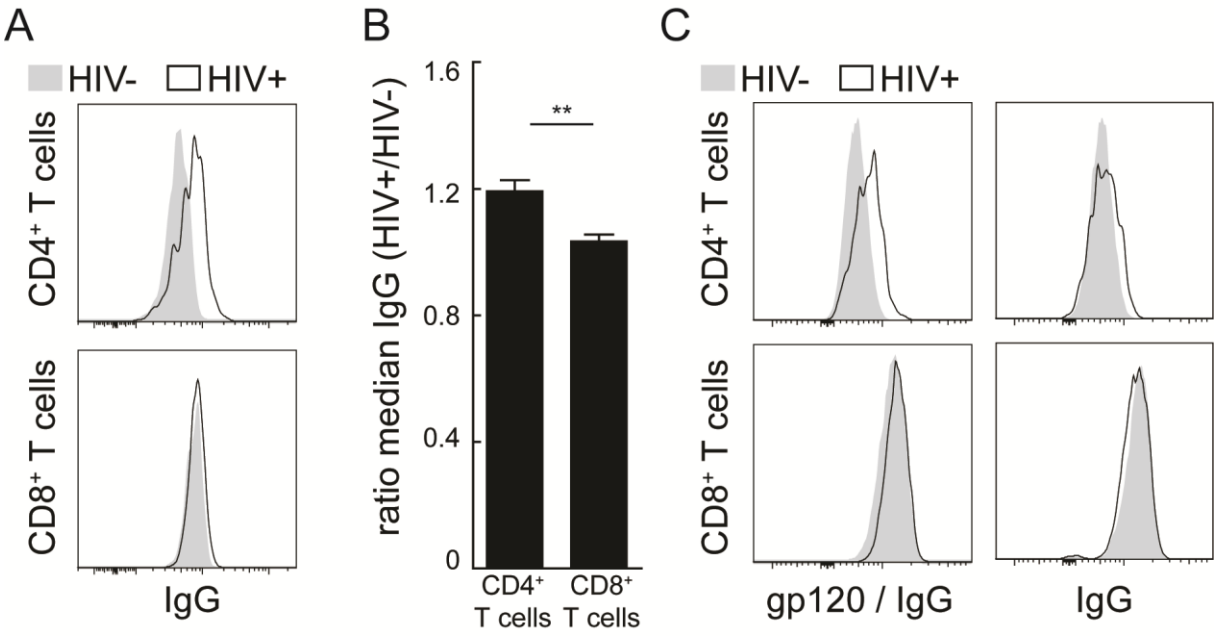

Figure 1: CD4⁺ T cells from HIV+ donors are covered with ICs which might at least partially contain gp120.

(A) Anti-human IgG antibody staining was performed on $CD4^+$ and $CD8^+$ T cells in blood from HIV-1^+ and HIV-1^- donors. Flow cytometry histogram shows human IgG staining of one representative experiment. (B) Bar graph depicting MFI in AU of human IgG on cells isolated from HIV-1^+ (n = 6) and HIV-1^- donors (n = 6) (n = nr of individual donors, $^{**}p < 0.01$, two-tailed unpaired t-test). (C) Anti-human IgG in combination with or without pre-incubation with human anti-gp120 mAb (clone 2G12 and 1-79) mAbs was performed on $CD4^+$ and $CD8^+$ T cells in blood from HIV-1^+ and HIV-1^- donors. Flow cytometry histogram depicts human gp120 / IgG staining or IgG staining of a preliminary experiment.

ICs are attached to CD4 receptors on $CD4^+$ T cells from HIV-1 patients

Next, we investigated whether these ICs on the surface of $CD4^+$ T cells from HIV-1 patients indeed were molecularly linked to CD4 receptors. Therefore, the levels of IgG on the surface of $CD4^+$ T cells from HIV-1 patients was investigated before and after they had been exposed to PMA to induce CD4 receptor internalization (Pelchen-Matthews, Parsons et al. 1993). We show that the level of anti-human IgG on $CD4^+$ T cells isolated from blood of HIV-1 patients indeed was reduced upon CD4 receptor down-regulation (Figure 2) what indicates that at least a proportion of ICs found on the surface of $CD4^+$ T cells is molecularly linked to the CD4 receptors.

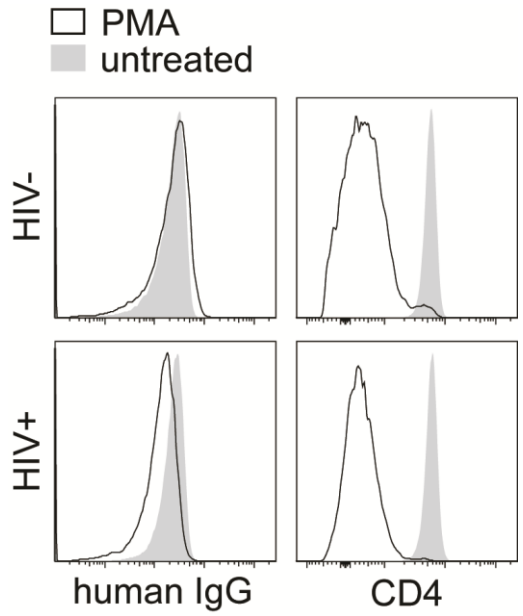

Figure 2: PMA mediated down-regulation of CD4 receptors is associated with decreased levels of IgG.

Anti-human IgG antibody staining was performed on $CD4^+$ T cells from blood of HIV-1^+ and HIV-1^- donors that were treated with PMA (50 ng/ml) for 6 hours. Flow cytometry histogram shows human IgG and CD4 receptor staining of a preliminary experiment.

The repertoire of anti-gp120 antibodies in the plasma from HIV-1 patients supports the formation of gp120-anti-gp120 ICs which bind to $CD4^+$ T cells and constrain their TCR-induced activation

Finally, we addressed the question whether the repertoire of polyclonal anti-gp120 antibodies present in the plasma of HIV-1 patients would support the formation of gp120-anti-gp120 ICs which would interfere with TCR-mediated $CD4^+$ T cell activation. To this end, plasma from HIV-1 patients was incubated with soluble gp120 to allow the generation of gp120-anti-gp120 ICs, followed by adsorption to $CD4^+$ T cells from healthy donors. Staining for anti-human IgG revealed that even in plasma from HIV^+ donors with high titers of anti-gp120 antibodies targeting the CD4BS (Table 1), such ICs could be detected on $CD4^+$ T cells, whereas plasma from healthy controls did not lead to the formation of ICs which specifically bound to $CD4^+$ T cells (Figure 3A). If such gp120 ICs composed of polyclonal antibodies from HIV^+ patients, in combination with anti-human CD3 mAb, had been loaded on $CD14^+$ monocytes, the activation of autologous $CD4^+$ T cells was impaired as shown by the analysis of CD69 expression after 6 hours (Figure 3B).

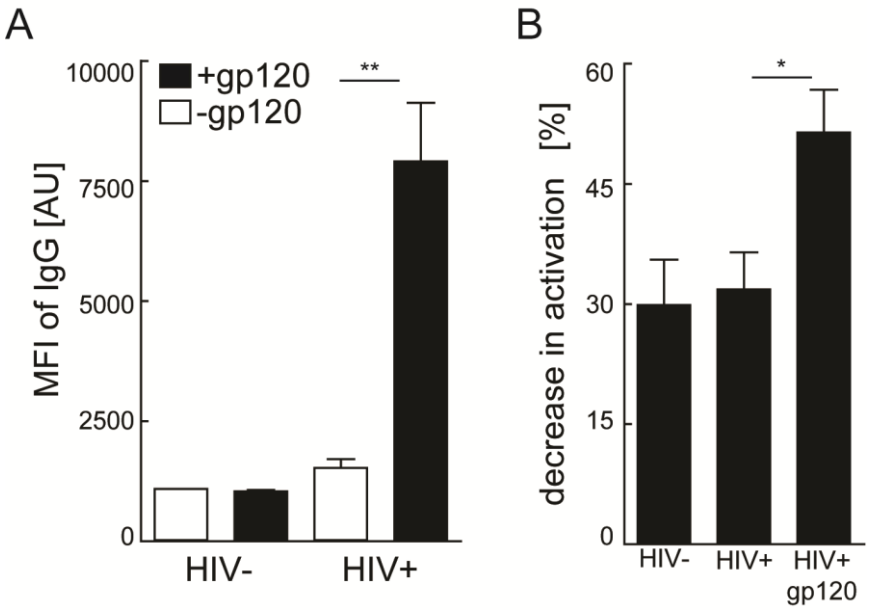

Figure 3: Anti-gp120 antibodies in the plasma from HIV-1 patients enable the formation of gp120-anti-gp120 ICs which bind to CD4⁺ T cells what impairs their activation.

(A) $CD4^+$ T cells from $HIV\text{-}1^-$ donors were incubated with plasma from $HIV\text{-}1^-$ (n = 5) or $HIV\text{-}1^+$ donors ± gp120 (n = 5). Bar graph showing MFI of IgG in AU as measure for IC (n = plasma samples from different donors, $^{**}p < 0.01$, one-tailed, paired t-test). (B) $CD4^+$ T cells were stimulated with monocytes loaded with αCD3 in combination with plasma from $HIV\text{-}1^-$ and $HIV\text{-}1^+$ donors ± gp120. Bar graphs represent the relative decrease in activation (measured by CD69 expression after 6 hours) induced by αCD3 in combination with plasma from $HIV\text{-}1^-$ donors (n = 7), $HIV\text{-}1^+$ donors (n = 16) or $HIV\text{-}1^+$ donors + gp120 (n = 16) compared to activation induced by αCD3 alone (n = nr of plasma samples from 5 different donors in several independent experiments, $^{*}p < 0.05$, one-tailed, paired t-test).

DISCUSSION

A peculiarity of HIV-1 is that its envelope surface glycoprotein has a very high affinity for the CD4 receptor (Myszka, Sweet et al. 2000), enabling HIV-1 and/or gp120 to directly interact and potentially interfere with $CD4^+$ T cell function. HIV-1 gp120' capacity to directly weaken $CD4^+$ T cell function was shown previously by us and others (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009). This effect has mostly been shown *in vitro* using relatively high concentrations of gp120, which leads to the question whether these effects might similarly operate in an *in vivo* scenario. Gp120 can be shed from the virion or from infected cells (Schneider, Kaaden et al. 1986, Pyle, Bess et al. 1987) and is detectable in the plasma of HIV-1 patients, albeit there is some uncertainty about the actual gp120 concentrations (Klasse and Moore 2004). Levels in the plasma, however, might be much lower compared to the ones in lymphoid tissues where gp120 is believed to accumulate preferentially (Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009) and at the same time represent the anatomic sites where the majority of $CD4^+$ T cells reside (Pantaleo, Graziosi et al. 1994) what implicates that $CD4^+$ T cells are exposed to high amounts of gp120 *in vivo* and thereby emphasizes gp120's role as mediator of $CD4^+$ T cell dysfunction *in vivo.* The robust antibody response against the envelope surface structure of HIV-1 (Lyerly, Reed et al. 1987, Nara, Garrity et al. 1991, Doria-Rose, Klein et al. 2009, McMichael, Borrow et al. 2010, McCoy and Weiss 2013) results in the formation of gp120-anti-gp120 ICs which are the most abundant form of gp120 *in vivo* (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1993). Consistent with this notion, we and others (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1993, Daniel, Susal et al. 1996, Daniel, Melk et al. 1999, Daniel, Sadeghi et al. 2004) demonstrate that $CD4^+$ T cells isolated from the blood of HIV-1 patients are covered with ICs, based on increased the IgG deposition on $CD4^+$ T cells compared to healthy controls. These ICs were identified to contain gp120 (Suzuki, Gatanaga et al. 2014) what we confirmed in preliminary experiments in which IgG levels on the surface of $CD4^+$ T cells were substantially elevated upon inclusion of gp120-specific human antibodies. Furthermore, we and

others (Suzuki, Gatanaga et al. 2014) demonstrate that these ICs on the surface of $CD4^+$ T cells from HIV-1 patients are molecularly linked to CD4 receptors since surface IgG levels are decreased upon selective down-regulation of CD4 receptors by PMA treatment (Pelchen-Matthews, Parsons et al. 1993). In addition, these effects were only relevant for $CD4^+$ T cells from HIV-1 patients and not for $CD8^+$ T cells what makes it conceivable that the increased IgG signal on $CD4^+$ T cells, at least partially, originates from a selective binding of gp120 containing ICs to the CD4 receptors. Consequently, similar as in our *in vitro* experiments in which $CD4^+$ T cells efficiently capture gp120-anti-gp120 ICs from APCs, we suggest that gp120-anti-gp120 ICs trapped on host cells via Fc- or complement-receptors as well as soluble gp120-anti-gp120 ICs might be effectively adsorbed on innocent bystander $CD4^+$ T cells. This could provoke hypo-responsiveness to subsequent TCR-induced activation and thereby add to $CD4^+$ T cell dysfunction during HIV-1 infection. Based on our *in vitro* experiments, we suggest that gp120-anti-gp120 IC coated $CD4^+$ T cells would by no means induce complete unresponsiveness to any TCR-induced stimulation but rather impedes with the TCR signaling pathway which is crucial in allowing $CD4^+$ T cells to adopt their proper functionality. This goes in line with previous reports disclosing that the activation potential of $CD4^+$ T cells isolated from HIV-1 patients is severely impaired compared to $CD4^+$ T cells isolated from healthy controls (Schnittman, Lane et al. 1990, Daniel, Susal et al. 1993, Meyaard, Otto et al. 1994). On top of that, since gp120 is predominantly found in lymphoid tissues (Klasse and Moore 2004, Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009), $CD4^+$ T cell dysfunction might be more pronounced in these anatomical compartments compared to the peripheral blood.

Importantly, gp120-anti-gp120 ICs generated by the addition of gp120 to plasma from HIV-1 patients shows that the repertoire of *in vivo* circulating polyclonal gp120-specific antibodies has the potential to generate gp120-anti-gp120 ICs which can be adsorbed by $CD4^+$ T cells. Antibodies which target the evolutionary conserved CD4BS (Scheid, Mouquet et al. 2011, Wu, Zhou et al. 2011, Kwong and Mascola 2012, Lynch, Tran et al. 2012) might prevent gp120-containing ICs from binding to CD4 receptors and hence not support gp120-related functional impairment of $CD4^+$ T cells similar as our ctrl IC. Our data suggest, however, that the presence of high CD4BS antibody titers in polyclonal plasma from HIV-1^+ donors does not prevent gp120-anti-gp120 IC formation upon gp120 addition and that such generated gp120 containing IC bind to $CD4^+$ T cells and negatively affect anti-human CD3 mAb-induced $CD4^+$ T cell activation. This might be

explained by the effective ratio of CD4BS versus non-CD4BS anti-gp120 antibodies in the circulation, with a low ratio still allowing the formation of gp120 containing IC capable to bind to CD4 receptors. Of note, plasma in which anti-gp120 antibody titers were below detection limit did not support the formation of gp120 containing ICs on $CD4^+$ T cells upon addition of gp120 (data not shown).

This study demonstrates that $CD4^+$ T cells from HIV-1 patients are covered with gp120-containing ICs which amongst other mechanism might contribute to the progressing decline of $CD4^+$ T cell function during HIV-1 infection.

MATERIALS AND METHODS

Ethics

Patients were enrolled in the Swiss HIV Cohort Study (Schoeni-Affolter, Ledergerber et al. 2010) or the Zurich Primary HIV infection study at the Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich (http://clinicaltrials.gov, ID 5 NCT00537966, (Rieder, Joos et al. 2011)). Approval and written informed consent from patients were obtained according to the guidelines of the Ethics Committee of the University Hospital Zurich.

Cell preparation

Citrate-phosphate-dextrose anti-coagulated buffy coats of healthy controls were purchased from the Swiss Red Cross (Blutspende Zurich, Switzerland) and EDTA anti-coagulated blood was obtained from HIV positive individuals from the University Hospital Zurich, Switzerland. Viral load in HIV-1 positive blood was determined at the Institute for Medical Virology at the University of Zurich (see Table 1 for detailed information). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphocyte Separation Media (LSM; PAA Laboratories, Pasching, Austria). Cells were washed in PBS and resuspended in RPM-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all reagents from PAA Laboratories, Pasching, Austria) what is referred to as RPMI-10 throughout the manuscript. If PBMCs were not directly used for experiments, they were cryopreserved in RPMI-1640 supplemented with 20% FBS and 10% DMSO (Sigma-Aldrich, Buchs, Switzerland). Cell viability was determined by 0.4% trypan blue exclusion (Invitrogen, Basel, Switzerland) and assessed to be greater than 90%. $CD4^+$ T cells and $CD14^+$ monocytes were isolated from PBMCs using anti-CD4 and -CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction.

Plasma samples

Plasma samples from HIV-1 patients (See Table 2 for detailed information) were heat inactivated at 56° C for 60 minutes and stored at -80° C until processing. Plasma viral loads (copies/ml), IgG anti-gp120 (JR-FL) titer as well as IgG anti-gp120-CD4BS titer were determined as described previously (Rusert, Kuster et al. 2005).

IC preparation

For the generation of gp120 containing ICs with plasma from HIV-1$^+$ donors, plasma from HIV-1$^-$ and HIV-1$^+$ donors and recombinant HIV-1 gp120 JR-FL (endotoxin level <1 EU/mg, Progenics Pharmaceuticals, Tarrytown, NY, USA) were both diluted 1 to 100 and incubated for at least 2 hours.

PMA induced down-regulation of CD4 receptors

CD4$^+$ T cells were stimulated with PMA (50 ng/ml, Sigma Aldrich Chemie, Buchs, Switzerland) for 6 hours.

In vitro CD4$^+$ T cell stimulation assay with autologous monocytes in the presence of ICs generated with plasma from HIV-1$^+$ donors

2×10^5 CD4$^+$ T cells were stimulated with 1×10^5 autologous CD14$^+$ monocytes in round-bottom 96 well cell culture plates in a final volume of 100 µl RPMI-10. Monocytes were loaded by sequential incubation of anti-human CD3 mAb (0.5 to 10 ng/ml, clone OKT3, eBioscience, Vienna, Austria) for 30 minutes at 4° C followed by incubation with gp120 containing ICs generated with plasma from HIV-1$^-$ and HIV-1$^+$ donors (as described under IC preparation) for 30 minutes at 4° C. Expression of CD69 was analyzed after 6 hours as described under "Flow cytometric analysis".

Flow cytometric analysis

Cells were surface stained with fluorochrome conjugated anti-CD3 (clone UHCT-1, BioLegend, Lucerna Chem AG, Luzern, Switzerland), anti-CD4 (clone SK3, BD Biosciences, Allschwil, Switzerland) and anti-human IgG (H+L) (Jackson Immunoresearch, Suffolk, UK) for 30 minutes at 4° C. If staining was performed in whole blood (Figure 7A and 7B), red blood cells were lysed with lysing solution (BD Biosciences, Allschwil Switzerland) directly after staining for 10 minutes at RT. For compensation, a combination of anti-mouse Igκ/Negative Control Compensation Particles (BD Biosciences, Allschwil, Switzerland) or PBMCs were used. Cells were resuspended in PBS containing 1% PFA (Sigma Aldrich Chemie) before acquisition. Acquisition was done on a LSRII Flow Cytometer (BD Biosciences, Allschwil, Switzerland) using FACS DIVA software. Samples were acquired on the day of the analysis and usually $1\text{-}3 \times 10^5$ cells of interest were acquired. Doublets were excluded using side- and forward-scatter height and width parameters, negative biological or fluorescence minus one (FMO) controls were used to set gates. Data analysis was done with FlowJo software (TreeStar, San Carlos, CA, USA).

Statistical analysis

Data was analyzed and plotted with GraphPad Prism (GraphPad, La Jolla, CA, USA) and results are illustrated as mean ± standard error of the mean (SEM). Statistical analysis was performed as specified in the figure legend. P-values less than 0.05 were considered statistically significant.

TABLES

Table 1. Characteristics of HIV-1 infected individuals.

Patient ID	Gender	Time period of HIV-1 infection	$CD4^+$ T cell count/µl	pVL copies/ml
1	m	< 5 years	20	290000
2	m	< 5 years	114	50200
3	m	< 5 years	359	257443
4	m	< 5 years	579	38987
5	m	> 10 years	314	22558
6	m	> 10 years	209	27208

Table 2. Characteristics of plasma samples from HIV-1 patients.

Patient ID	IgG anti-gp120 (JR-FL) titer	IgG anti-gp120-CD4BS titer	Plasma viral load (copies/ml)
1_1	15107	6682	22510
1_2	14166	4926	283140
1_3	7342	5590	53500
2_1	16.54	1*	29700
2_2	8.23	1*	32800

* Values <1 indicate undetectable levels of anti-gp120-CD4BS antibody titers

General Discussion

VIRUS MEDIATED MODULATION OF TCR SIGNALING

Viral replication must outbalance anti-viral immunity (Virgin, Wherry et al. 2009) in order to establish a successful infection. Immune responses directed against the infecting virus critically depend on T cells and since the T cell receptor (TCR) signaling pathway is crucial in dictating T cell functionality, it is not surprising that many viruses evolved to interfere with TCR signaling. The enhancement of TCR signaling to provide more activated cellular targets for viral replication and in contrast, the impairment of TCR signaling as a mechanism of immune evasion are likely scenarios pursued by viruses that evolved to modulate TCR signaling (Jerome 2008).

THE TCR SIGNALING PATHWAY IS CRUCIAL IN REGULATING ADAPTIVE IMMUNITY

The exertion of T cell effector function is the basis of a functional adaptive immune system which allows the elimination of invading pathogens and maintenance of homeostasis (Smith-Garvin, Koretzky et al. 2009). Since the specificity as well as effector functions of T cells rely on the engagement of TCRs with MHC bound peptide complexes on antigen-presenting cells (APC) or target cells, any interference with TCR signaling could easily switch the balance in favor of the infecting virus. TCR engagement induces the recruitment and activation of the protein tyrosine kinase p56lck which is non-covalently associated with the CD4 receptor (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991, Filipp, Leung et al. 2004, Kusumi, Ike et al. 2005) leading to tyrosine phosphorylation of immunoreceptor-based tyrosine activation motifs (ITAMs) on the ζ, γ, δ, and ε chains of the TCR/CD3 complex. Phosphorylated ITAMs allow the recruitment and activation of Zap-70 which in turn leads to the phosphorylation of LAT and SLP-76 what serves as scaffold to recruit further signaling molecules that regulate T cell activation, proliferation and differentiation (reviewed by Brownlie and Zamoyska (Brownlie and Zamoyska 2013)). Many molecules are known to positively or negatively enhance TCR signaling, the main co-stimulator of T cell activation is CD28 (Riley and June 2005) which competes with CTLA-4 (Teft, Kirchhof et al. 2006) for the same ligands what would in contrast lead to decreased levels of TCR signaling.

HIV INTERFERES WITH TIGHTLY CONTROLLED TCR SIGNALING PATHWAY

$CD4^+$ T cells are the main site of HIV replication *in vivo* which represent simultaneously the cell type that coordinates HIV-specific immunity and it is therefore not surprising that HIV has evolved several strategies to modulate TCR signaling. A beneficial environment for viral replication could be generated by positively or negatively modulating TCR signaling and hence the activation of the $CD4^+$ T cells. Namely, increased $CD4^+$ T cell activation provides more susceptible target cells for efficient viral propagation and production of progeny virus based on the fact that the efficacy of HIV-1 replication in $CD4^+$ T cells is tightly coupled to their activation state (Schnittman, Lane et al. 1990, Zack, Arrigo et al. 1990, Bukrinsky, Stanwick et al. 1991). In contrast, a reduction of the T cell mediated anti-viral immune response (Munier, Kelleher et al. 2013) by direct interference at the level of optimal TCR stimulation would also promote further HIV-1 replication, as T cell effector functions would be down-modulated. HIV mediated control of TCR signaling is by no means a simple binary event, but rather a complex process which - at least conceptually - might profit from negative and positive interference with TCR signaling. HIV-1 proteins were shown to have strong TCR modulating capacity, for example the accessory HIV-1 protein Nef was shown to modulate TCR signaling in a positive manner by enhancing levels of IL-2 (Schrager and Marsh 1999), activation of NFAT (Manninen, Renkema et al. 2000) or by the induction of a transcriptional profile reflective for TCR engagement (Simmons, Aluvihare et al. 2001). Several reports however, rather suggest that HIV-1 Nef inhibits T cell activation (Niederman, Hastings et al. 1993, Bandres and Ratner 1994), either by inducing a down-regulation of the CD4 receptor (Craig, Pandori et al. 1998) or by driving the accumulation of TCR and p56lck in the recycling endosomal compartment and thereby preventing the clustering of signaling molecules beyond the site of TCR engagement (Thoulouze, Sol-Foulon et al. 2006). In line with these contradictory findings, a dual function was attributed to HIV-1 Nef in modulating TCR signaling of infected $CD4^+$ T cells, whereas HIV-1 Nef leads to increased activation of quiescent $CD4^+$ T cells by the enhanced activation of NFAT, HIV-1 Nef induces $CD4^+$ T cell unresponsiveness in pre-activated cells by impairing PLC-γ activation (Neri, Giolo et al. 2011). Furthermore, HIV-1 Tat was shown to have an inhibitory effect on TCR-induced activation and proliferation (Viscidi, Mayur et al. 1989). The very prominent effect of HIV-1 gp120 on the modulation of TCR signaling is discussed in the next section.

HIV-1 GP120 INTERFERES WITH CD4+ T CELL FUCTION BY BINDING TO THE CD4 RECEPTOR

CD4 RECEPTORS ARE KEY DETERMINANTS IN CONTROLLING TCR SIGNALING EFFICIENCY

CD4 receptors are transmembrane glycoproteins of the immunoglobulin superfamily which are expressed on MHC class II restricted T cells and on cells from the macrophage/monocyte lineage. CD4 receptors play a crucial role in TCR signaling since TCR–pMHC interactions alone do not efficiently trigger T cell activation, but require the participation of CD4-pMHC interaction which significantly potentiates TCR binding and signaling efficiency (Xiong, Kern et al. 2001). The principal role of the CD4 molecules is to increase avidity between T cells and APCs as well as direct contribution in signal transduction through the recruitment of the non-covalently associated src tyrosine kinase p56lck (Rudd, Anderson et al. 1989, Veillette, Bolen et al. 1989, Glaichenhaus, Shastri et al. 1991) to the site of TCR–pMHC complex engagement (Ledbetter, June et al. 1988, Rudd, Anderson et al. 1989, Veillette, Bookman et al. 1989, Glaichenhaus, Shastri et al. 1991, Filipp, Leung et al. 2004, Li, Dinner et al. 2004, Kusumi, Ike et al. 2005). Consequently, any interference with CD4 receptor engagement would modulate TCR-induced signaling what can be shown experimentally by antibody induced cross-linking of the CD4 receptor which results in a tyrosine phosphorylation pattern reflective of an activated TCR signaling pathway (Veillette, Bolen et al. 1989).

HIV-1 GP120-CD4 RECEPTOR INTERACTION IS NOT RESTRICTED TO THE INDUCTION OF VIRAL ENTRY

The basis for the complex interplay between HIV-1 and $CD4^+$ T cells lies in HIV's surface envelope glycoprotein gp120 which has a very high affinity for the CD4 receptor (Myszka, Sweet et al. 2000) and allows human and simian immunodeficiency viruses to specifically interact with $CD4^+$ T cells. Gp120-CD4 receptor interaction represents the first step of viral entry (Dalgleish et al, 1984; Sattentau, 1988) which ultimately leads to the infection of the cells. The fact however, that only a limited fraction of circulating virions are demonstrably infectious (Dimitrov, Willey et al. 1993) and only a very small proportion of $CD4^+$ T cells is productively infected (Harper, Marselle et al. 1986, Haase, Henry et al. 1996, Chun, Carruth et al. 1997, Anderson, Ascher et al. 1998, Haase 1999, Douek, Picker et al. 2003) compared to the considerable

decline of CD4+ T cell function in a quantitative and qualitative manner (Clerici, Stocks et al. 1989, Dolan, Clerici et al. 1995, Hazenberg, Hamann et al. 2000, McCune 2001, Grossman, Meier-Schellersheim et al. 2002, Guadalupe, Reay et al. 2003, Brenchley, Schacker et al. 2004, Mehandru, Poles et al. 2004, Gordon, Cervasi et al. 2010, Okoye and Picker 2013) suggests that gp120-CD4 receptor interaction not only mediates direct infection of the cells but might also interfere with TCR signaling. This could substantially contribute to the dysregulated CD4+ T cell response during HIV infection.

HIV-1 GP120 MEDIATED INFLUENCE ON TCR-INDUCED ACTIVATION OF CD4+ T CELLS

Many studies indeed showed that gp120 influences TCR signaling in CD4+ T cells, be it in a negative (Chirmule, Kalyanaraman et al. 1988, Diamond, Sleckman et al. 1988, Kornfeld, Cruikshank et al. 1988, Mittler and Hoffmann 1989, Weinhold, Lyerly et al. 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Manca, Habeshaw et al. 1990, Oyaizu, Chirmule et al. 1990, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Hivroz, Mazerolles et al. 1993, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994, Chirmule, Goonewardena et al. 1995, Chirmule, McCloskey et al. 1995, Marschner, Hunig et al. 2002, Nyakeriga, Fichtenbaum et al. 2009) or a positive manner in terms of increased calcium signaling and IL-2R expression (Kornfeld, Cruikshank et al. 1988), transiently enhanced p56lck activity (Juszczak, Turchin et al. 1991, Hivroz, Mazerolles et al. 1993), activation of the transcription factors AP-1 (Chirmule, Goonewardena et al. 1995) and elevated proliferation (Oravecz and Norcross 1993). CD4+ T cell dysfunction was suggested to be induced through gp120's induction of CD4 receptor endocytosis (Chirmule, Kalyanaraman et al. 1988, Juszczak, Turchin et al. 1991, Cefai, Ferrer et al. 1992, Schwartz, Alizon et al. 1994, Theodore, Kornfeld et al. 1994), the prevention of peptide-MHCII-CD4 receptor interaction (Diamond, Sleckman et al. 1988, Rosenstein, Burakoff et al. 1990), the abolishment of proximal TCR signaling (Mittler and Hoffmann 1989, Cefai, Debre et al. 1990, Chirmule, Kalyanaraman et al. 1990, Oyaizu, Chirmule et al. 1990, Goldman, Jensen et al. 1994) or the redistribution of p56lck away from TCRs or from the IS (Goldman, Crabtree et al. 1997, Nyakeriga, Fichtenbaum et al. 2009).

The capacity of non-infectious gp120 to modulate CD4+ T activation was convincingly demonstrated in these studies, however whether gp120 does this in a positive or negative manner *in vivo* remains elusive. Therefore, we aimed in this thesis to reinvestigate the capacity of gp120 to interfere with the TCR signaling

pathway. Our data confirms that a selective binding of gp120 to CD4 receptors on human $CD4^+$ T cells can either significantly enhance or diminish TCR-induced $CD4^+$ T cell activation and we demonstrate that these opposing effects can be explained by the relative orientation of CD4 receptor and TCR engagement. Synchronous and spatially linked CD4/TCR engagement by plate-immobilized gp120 or gp120 ICs in combination with low amounts of agonistic anti-CD3 mAbs promotes full activation of $CD4^+$ T cells. Thus, in a situation where the amount of agonistic anti-CD3 mAbs is not sufficient to exceed local thresholds for efficient TCR signaling propagation, concomitant CD4 receptor cross-linking by gp120 at the site of TCR engagement provides additional stimulation which together lead to full $CD4^+$ T cell activation. This attributes gp120 a crucial role in enhancing low levels of TCR signaling efficiency. In contrast to that, $CD4^+$ T cells became hypo-responsive to stimulation by relative high levels of agonistic anti-CD3 mAbs upon interaction with anti-CD3 mAbs / gp120 immune complex (IC) loaded monocytes. Importantly, the close proximity of $CD4^+$ T cells and gp120 IC loaded monocytes leads to an efficient transfer of gp120 ICs from monocytes to $CD4^+$ T cells whose CD4 receptors are consequently decorated with gp120 ICs. We show that gp120-anti-gp120 IC binding to CD4 receptors impairs the formation of IS beyond the engaged TCR by tethering CD4 receptor associated p56lck away from the engaged TCR and by hindering the cytoskeleton dependent accumulation of signaling molecules through the physical association of CD4 receptors with elements of the cytoskeleton. In a situation however, in which CD4 receptors are cross-linked selectively at the site of TCR engagement, the IS formation is facilitated by CD4-mediated recruitment and activation of p56lck to the site of stimulation. These findings resolve the reported controversies on how gp120 affects $CD4^+$ T cell responsiveness to TCR stimulation and demonstrates a very prominent way of how HIV interferes with the functionality of $CD4^+$ T cells at the level of the TCR signaling pathway. Importantly, a scenario in which $CD4^+$ T cells are exposed to gp120 ICs on the surface of monocytes, macrophages or dendritic cells is much more likely of physiological relevance compared to a situation in which TCR engagement and CD4 receptor cross-linking are provided in a spatially linked manner as is the case by plate-immobilized stimuli.

In an attempt to underline the importance of gp120-CD4 receptor interaction in inducing immune dysfunction, gp120 levels in the circulation or lymphoid tissues were measured in HIV-1 patients or SIV infected non-human primates. Gp120 is readily shed from the virion surface (Gelderblom, Reupke et al. 1985, Schneider, Kaaden et al. 1986, Pyle, Bess et al. 1987) and occurs preferentially in the presence of

CD4 receptor or antibody binding (Moore, McKeating et al. 1990). Consequently, gp120 was detected in the circulation (Gilbert, Kirihara et al. 1991, Oh, Cruikshank et al. 1992, Rychert, Strick et al. 2010) and in higher concentrations in lymphoid tissues (Popovic, Tenner-Racz et al. 2005, Santosuosso, Righi et al. 2009) even in the absence of detectable viral replication. These studies demonstrate that gp120 is indeed present in the serum or lymphoid tissues of HIV-1 patients, the amount however varied considerably between studies, probably reflecting different methodologies (e.g. different sensitivities of gp120 ELISA) (Klasse and Moore 2004), adsorption of gp120 to proteoglycans on cell surfaces (Mbemba, Benjouad et al. 1999) or masking of gp120 by antibodies (Daniel, Susal et al. 1998). It is therefore still elusive whether or to what extent gp120 has indeed the potential to influence immune cell function *in vivo* and whether *in vitro* experiments based on exogenous gp120 treatment are indeed reflective for an *in vivo* situation. Another concern might be the fact that gp120 is heavily glycosylated *in vivo* which might be different from recombinantly produced gp120 whose glycosylation pattern depends on the cellular expression system used (Mizuochi, Matthews et al. 1990). It was however shown that glycosylation is in fact necessary for proper protein folding, but not for its interaction with the CD4 receptor (Bahraoui, Benjouad et al. 1992) what makes it unlikely that the gp120 glycosylation pattern prevents a CD4-mediated effect on $CD4^+$ T cell function. Experimental evidence by us and others (Amadori, De Silvestro et al. 1992, Daniel, Susal et al. 1993, Daniel, Susal et al. 1998, Suzuki, Gatanaga et al. 2014) demonstrate the presence of ICs on the surface of $CD4^+$ T cells from HIV-1 patients. Since these ICs are molecularly linked to the CD4 receptor (Suzuki, Gatanaga et al. 2014) and might at least partially contain gp120, gp120 in the form of gp120 ICs might indeed play a crucial role in modulating $CD4^+$ T cell activation *in vivo*.

CONCLUDING REMARKS

The high affinity of gp120 to the CD4 receptor provides a prominent way for HIV's to interfere with the activation of $CD4^+$ T cells by modulating their TCR signaling pathway. We suggest that gp120 containing ICs render $CD4^+$ T cells from HIV-1 patients hypo-responsive to subsequent TCR stimulation and based on the importance of $CD4^+$ T cells in coordinating adaptive immune responses, such gp120-mediated down-regulation of the $CD4^+$ T cell functionality might substantially contribute to the progressive immunodeficiency during HIV-1 infection. Along that line, blocking of the CD4-gp120 receptor interaction is considered as promising therapeutic option for HIV-treatment in order to maintain $CD4^+$ T cell functionality

(Vermeire and Schols 2005). However, the importance of CD4 receptors in regulating $CD4^+$ T cell function via the TCR signaling pathway might provoke strong adverse immunomodulatory effects upon administration of such CD4 receptor targeting agents and thereby limit their benefit in clinical applications (Horneff, Burmester et al. 1991, Horneff, Guse et al. 1993, Olive and Mawas 1993).

On top of gp120's capacity to modulate TCR-induced activation of $CD4^+$ T cells and inducing viral entry into host cells, gp120 binding to diverse immune cells induces a plethora of biological effects such as maturation, unresponsiveness or cell death which are extensively reviewed in Chirmule &Pahwa (Chirmule and Pahwa 1996) as well as Conti and colleagues (Conti, Fantuzzi et al. 2004). Although a possible profound disturbance of the immune system is implied from these studies, the actual *in vivo* effect remains elusive. Therefore, a very challenging goal of future HIV research is to translate the wealth of theoretical and empirical models into physiologically more relevant ones which would allow a more complete understanding of HIV-1 pathogenesis.

Abbreviations

AICD	Activation induced cell death
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
ART	Antiretroviral therapy
CCR5	CC chemokine receptor 5
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CXCR4	CCX chemokine receptor 4
DAPI	6′ -diamidino-2-phenylindole
DC	Dendritic cell
DMSO	Dimethylsulfoxide
EBV	Epstein-Barr Virus
EDTA	Ehylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcR	Fc receptor
HSV	Herpes simplex virus
IC	Immune complex
IS	Immunological Synapse
LPS	Lipopolysaccharide
LTNP	Long-term non progressors
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
MTOC	Microtubule organizing centre
p56lck	Lymphocyte cell-specific protein-tyrosine kinase 56
pDC	plasmacytoid DC
PBMC	Peripheral blood mononuclear cell
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus
TCR	T cell receptor

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