（2）HIV 感染症における病状進行速度の臨床検討

研究要旨：
2006―2009 年の HIV 感染症 15 例の免疫不全進行速度を検討した．従来報告されているより近年の症例は進行速度が加速している可能性が示唆された．今後の課題としてウイルス、宿主双方の免疫不全進行速度に関わる因子についての検討を重ねて行きたい。

A. 研究目的

従来は HIV 感染から 7 -10 年後に AIDS を発症すると考えられていたが，近年，AIDS 発症までの期間が短縮されているとの報告が増えている.

本研究の目的は琉球大学医学部附属病院第一内科において経験した急性 HIV 感染症の経過および HAART 導入までの期間を解析し、免疫不全の進行速度を明らかにすることである.

B. 研究方法

1. 感染から 4 年以内の症例の CD4 陽性 T リンパ球の推移

2006 年から 2009 年の 4 年間に当院で経験した HIV 感染症で、HIV 感染の時期が客観的に 4 年以内と推定できる症例に関して診療録を元にレトロスペクティブに CD4 陽性 T リンパ球の低下について解析を行った.

２．急性 HIV 感染症症例の CD4 陽性 T リンパ球数の推移

急性 HIV 感染症として診断し得た 9 症例の CD4 陽性 T リンパ球数の経過についてプロスペクティブに解析を行った.

倫理面への配慮

1)個人情報の取得について

利用目的を明確にしたうえで、必要とする範囲内に限り、適法かつ公正な手段によって、個人情報を取得した。

2)個人情報の利用について

個人情報を取得する際に示した本研究の範囲内で、研究の遂行上、必要な限りにおいて利用した。

3)個人情報の第三者提供について

取得した個人情報は、適切に管理し、個人情報保護法に定める例外事項を除き、本人の同意を得ることなく、第三者に提供、開示しなかった。

4)個人情報の開示・訂正・削除・利用停止について

本人から自己の個人情報について、開示・訂正・削除・利用停止などの申し出を受けた場合は、すみやかに必要な措置をとる旨説明したが、研究の全期間を通して申し出はなかった。

5)個人情報の管理について

個人情報の紛失、流出、改ざんおよび漏洩などを防ぐため、個人情報を保有するのは研究代表者のみとし、情報管理上問題は発生しなかった。

6)法令等の順守について

個人情報保護に関して適用される法令、国のガイドラインを熟読し順守した。

C. 結果

１．感染から 4 年以内の症例は 6 例の症

例が該当した (Table 1). CD4 数の中央値は 181.5cellc/μL (9−695/μL) で、6 例中 2 例はニューモシスチス肺炎を契機に AIDS を発症していた. 残り 4 例中 3 例に関しては感染から 4 年以内に CD4 数 < 200/cellsμL を呈しており HAART 導入となっていた(表　感染後 4 年以内の 6 症例).

表 HIV感染から4年以内の症例のCD4数

Pt No	最後にHIV抗体陰性を確認〜診断までの年数	病期	OI	CD4 cells/μL	HIV-RNA copies/mL
1	3年	AIDS	PCP	25	3.8×10^5
2	4年	AIDS	PCP	9	1.2×10^5
3	1年	AC	−	695	5.0×10^4
4	2年	AC	−	164	2.0×10^5
5	3年	AC	−	141	3.8×10^3
6	4年	AC	−	55	1.8×10^3

2. 急性 HIV 感染症 9 例の CD4 数の推移に関して図に示した. 9 例中 7 例は診断から 48 週以内に HAART 導入となっていた

(図　急性 HIV 感染症 9 例).

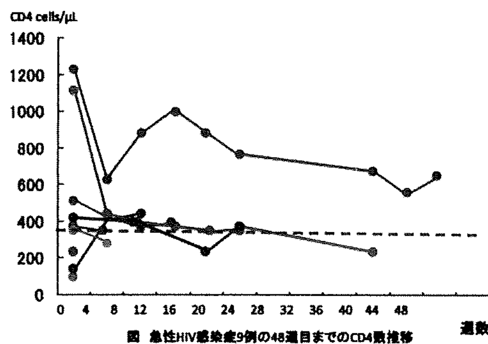

図 急性HIV感染症9例の48週目までのCD4数推移

D. 考察

今回の検討では、最近の HIV 感染症例の免疫不全進行速度が従来報告されているものより加速している可能性が示唆された.

HIV 感染の免疫不全進行速度に影響を与える因子として併発する性感染症などが報告されている. 宿主側の因子として、いくつかの HLA が HIV 感染に対して防御的であることもこれまで知られている. Kawashima らは日本人に多くやはり HIV 感染に対して防御的である HLA-B*51 に対する免疫逃避ウイルスが増加していることが、昨今の免疫不全進行速度に影響を与えている可能性を指摘している.

今回の我々の検討は少数例であったが、今後の課題としてさらなる症例数蓄積とウイルス、宿主双方の免疫不全進行速度に関わる因子についての検討を重ねて行きたい。

E. 結論

HIV 感染後, AIDS 発症までの期間または免疫能低下をきたすまでの期間が従来考えられていたより進行が早くなっている可能性が高い. これはウイルス量の多寡との関連は必ずしも相関は認められなかった.

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

(共同研究者；健山正男、仲村秀太, 田里大輔, 比嘉　太)

（3）AIDS 関連播種性 *Mycobacterium avium* 感染症の免疫学的機序の解明

研究要旨：
Mycobacterium avium 感染症の AIDS における病態は充分に明らかでない。我々は、播種性 *M. avium* 症感染モデルとしてのブタを用いた基礎的検討により *M. avium* は経口感染し、腸粘膜を経てリンパ行性および経門脈性に全身に播種することが示唆された。その上で AIDS 剖検症例を用いて検討を行った結果、腸管が侵入門戸であり、経リンパ行性に病巣が拡大したことが推察された。さらに免疫学的な検討により感染制御に重要な IFN-γや TNF-αの産生低下により菌の増殖、肉芽腫の形成不全に至り、播種が成立すると考えられた。

A. 研究目的

A．研究目的

Mycobacterium avium 感染症は、既往歴のない中高年女性を中心に環境中より経気道的に感染することで発症する呼吸器疾患の一つである。しかしながら HIV 感染症の流行とともに日和見的な全身感染の存在が明らかとなり、近年は、抗レトルウイルス薬による治療（HAART）の導入によりリンパ節炎などを主体とする免疫再構築に伴う *M. avium* 感染症が臨床的な重要性を増している。また AIDS 症例では経腸感染が主体であり、播種性であっても肺病変を形成する率は低いことが知られている。本研究の目的は、HIV 感染症に合併する *M. avium* 感染症の臨床病理学的解析を行うことを目的とする。

B. 研究方法

2002 年から 2004 年の間、沖縄県でと畜されたブタのうち、全身性に *M. avium* 感染による肉芽腫性病巣を認めた 276 個体、感染組織としては 3,312 検体（276 個体 x12 組織）を対象として用いた。全身感染の定義は、腸間膜リンパ節あるいは顎下リンパ節と肝臓あるいは脾臓や肺など実質臓器に感染病巣を認める個体とした。また AIDS 症例での検討に対しては、国立国際医療センターにて 2001 年から 2003 年までの間、剖検が行われた HIV 感染者のうち播種性 *M. avium* 感染が確認された 5 症例を対象とした。得られた組織は、一般的な処理により組織標本を作製し、病理組織学的検討に用いた。菌種の同定はアンプリコアマイコバクテリウムキット（日本ロッシュ社製）を用い、亜種の同定には遺伝子挿入配列 IS*1245* の制限断片長多型配列を解析することにより行った。

（倫理面への配慮）

剖検材料の使用に関しては、研究機関および国際医療センターの倫理委員会の承認を受け、規則に従い実施した。実際には、剖検時の承諾書により研究目的に組織を用いる事の同意がなされている。またヒト組織材料を使用するため、下記の点を留意して実験を行った。

1) 研究に用いる組織は、他の研究目的には使用しない。

2) カルテ等の個人情報に関しては、施設外に持ち出す際は、個人を特定を不可能とするための処置を行い、記号番号により管理した。

3) 本研究により、直接提供者が医学上の利益・不利益を得ることはない。

C. 研究結果

ブタに関する研究については論文としてまとまったのでPDFファイルを添付する。ブタでの検討では、病巣は腸間膜リンパ節と肝臓に98%以上の割合で感染病巣を認めた。しかし、腸管膜リンパ節の上位リンパ節には病巣を認めなかった。病巣を認めた器官における組織像は、約半数の個体で非特異的な滲出性病巣を示した。肝病巣に着目すると、その病巣は66%の個体（n=112）で門脈域に限局し、組織像が限界板壊死を伴った滲出性炎症像あるいは脾臓病変が存在する個体では有意に小葉内病巣を認めた。

このことからブタは *M. avium* に経腸感染し、腸間膜リンパ節に病巣を形成するとともに、門脈を経て肝臓の門脈域に入り、菌に対する宿主の防御反応としての炎症が肝組織の破壊をもたらし血行性に播種することが示唆された。このことを踏まえAIDS剖検症例で検討を行った。対象とした5例のうち多剤併用療法（HAART）導入前の症例は2例、導入後の症例は3例であった。いずれの症例もHAART導入期間中も全体として低いCD4値の推移を示した。全症例で抗結核薬の標準的な投与がなされていた。病変の組織像は、壊死を伴う被包化された肉芽腫が1例に認められた。4例は炎症細胞の浸潤に乏しい泡沫状組織球の集簇からなっていた。また3例に消化管病巣を認め、4例に腸間膜リンパ節およびその上位リンパ節である大動脈周囲リンパ節に病巣を認めた。肝臓においては1例を除き明らかな病変は門脈域に認められなかったものの、門脈域の結合組織内に存在した泡沫状組織球および拡張した類洞内の組織球に抗酸菌を多数認めた。何れの症例においても肺病変は認められなかった。さらに免疫組織化学染色により感染病巣でのリンパ球の各分子の発現状況を検討した。腸粘膜およびリンパ節病巣では、$CD4^{+}$細胞や$CD56^{+}$細胞の減少、T-betに比べGATA-3の優位な発現、$CD3^{+}$細胞に比べ$CD20^{+}$細胞の有為な存在を認めた。またリンパ節病巣ではFoxP3、SOCS3の発現増加が認められた。こうした病巣に集族しているリンパ球はHIVp24陽性であった。

D. 考察

免疫応答宿主の肺 *M. avium* 感染症においては、その組織像は類上皮細胞性肉芽腫の像を示し、時に乾酪性であることが知られている。一方、AIDS関連播種性MAC症では泡沫状組織球の集簇が特徴的所見であり、今回の検討においてもそれに準ずるものであった。壊死を伴う肉芽腫を示した1症例はHAART導入後であり、*M. avium* 診断時のCD4値は162μ/mlと症例中最も高値であった。このことから宿主の免疫能が組織像に影響したと考えられる。また消化管および腸間膜リンパ節や大動脈周囲リンパ節に病巣を認めたことから、腸管が侵入門戸であり、経リンパ行性に病巣が拡大したことが推察される。しかし、血中や骨髄において菌が証明されていることから、後に血行

性へと移行することが推測された。

HIV 感染では腸粘膜での $CD4^{+}$リンパ球の枯渇や NK 細胞の減少、機能不全が知られている。このことは菌の腸粘膜下での感染・増殖を容易にする。また SOCS3 や FoxP3 の発現増加は IL-12/IFN-γ 経路の抑制に繋がる。近年の研究により、HIV 産生蛋白がマクロファージの機能不全を引き起こす事も知られている。このことから感染制御に重要な IFN-γ や TNF-α の産生低下により菌の増殖、肉芽腫の形成不全に至り、播種が成立すると考えられた。しかし、本研究で検討された症例はいずれも抗結核薬および HAART が長期に渡り投与されていた。それにもかかわらず末梢の組織像や抗酸菌数が HAART が導入される以前の患者にみられる従来の組織像と変化がなかったこと、さらに HIV 感染リンパ球を認めた事は、HAART が十分に末梢組織に到達していないことが考えられ、そのことにより局所における細胞性免疫能の低下の改善が得られず、肉芽腫の形成不全、抗酸菌の増殖を加速したものと考えられた。

E. 結論

AIDS 関連播種性 MAC 症は播種に至る要因として、末梢組織における細胞性免疫能の極度の低下が原因であり、播種は第一にリンパ行性に起きることが組織学的に明らかとなった。

F. 健康危険情報

なし

G, 研究発表

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H. 知的財産権の出願・登録状況

なし

（共同研究者；日比谷健司、仲村秀太、田里大輔、山城剛、知念寛、比嘉太、健山正男、仲宗根勇、山根誠久、久場睦夫、鹿住祐子、菅原勇、稲垣考、市川和哉、小川賢二、西内由紀子、照屋勝治、菊池嘉、岡慎一、望月眞、遠藤久子、）

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表レイアウト

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ. 研究成果の刊行物・別刷

Impairment of *in vitro* generation of monocyte-derived human dendritic cells by inactivated HIV-1: involvement of type-I interferon produced from plasmacytoid dendritc cells

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Running title: Impairment of DC generation by HIV-1

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Abstract

In an attempt to simplify the protocol of DC generation *in vitro*, studies conducted herein show that functional DCs could be generated from bulk peripheral blood mononuclear cells (PBMCs) in media containing GM-CSF and IL-4. Interestingly, when PBMCs, but not purified monocytes, were exposed to either CCR5- or CXCR4-tropic inactivated HIV-1 isolates (iHIV-1) at the initiation of the culture, DC yields were significantly reduced in a dose-dependent manner due to monocyte apoptosis. Similar impairment of DC generation was noted with the use of type-I IFNs and poly I:C not only in cultures of PBMCs but also using highly enriched monocytes. This effect was reversed by anti-human type-I IFN receptor, but not by anti-FasL, anti-TRAIL, anti-TNF or a mixture of these antibodies. iHIV-1-exposed PBMCs, but not monocytes, produced high levels of IFN-α but not IFN-β. PBMCs depleted of $CD123^+$ plasmacytoid DCs produced low levels of IFN-α and were resistant to iHIV-1-mediated DC impairment. Interestingly, exogenously added TNF reversed the impairment by iHIV-1 in the PBMC cultures. In conclusion, the present results indicate that iHIV-1 impairs the *in vitro* generation of functional DCs from PBMCs through the induction of IFN-α from plasmacytoid DCs in a CD4-dependent fashion in the absence of TNF.

1. Introduction

The high potency of dendritic cells (DCs) in the processing and presentation of antigens to T cells *in vivo* has prompted their use as potential therapeutic antigen specific delivery vehicles to promote tumor- and virus-specific T-cell responses in patients with cancer and a variety of infectious diseases including HIV-1 [1-3]. These strategies were based on the successful employment of such techniques not only in murine but also in simian models [4]. Several laboratories including ours have also successfully utilized DC based HIV specific immunization protocols utilizing the humanized mouse model (hu-PBL-SCID) [5, 6]. Of importance was the finding that such DC-based immunizations were found to be safe in a number of vaccine trials using HIV-1 CD8-epitope peptides in humans [7]. However, implementing such DC based immunization protocols requires the isolation of significant numbers of DC's which in the case of untreated HIV-1 infection provides a challenge. Thus, decreased numbers and function of circulating myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) has been recorded as one of the hallmarks of untreated HIV infection [8-11]. In addition, for reasons unclear at present, there is poor reconstitution of pDC during ART [12, 13], which has been reasoned to contribute to the poor immune responses in these patients in trials that involved immunization with inactivated autologous HIV-1. The remarkable potency of peptide based DC vaccination thus holds great promise if methodologies can be identified to procure the required number of DCs for such immunization [14-16].

DCs for such clinical studies are obtained either directly from blood or generated *in vitro* from circulating $CD34^+$ hematopoietic stem cells [17]. More recently, however, it has become common to generate DCs from peripheral blood monocytes by *ex vivo* culture. The specific methods used for cell purification, culture and maturation vary widely. Commonly, monocytes are isolated from blood by adherence [18], elutriation [19] or positive or negative selection using immunomagnetic beads [20]. These enriched population of monocytes are then induced to differentiate into DCs in vitro using medium supplemented with granulocyte-macrophage–colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [21]. However, since the dose, quality of reagents, additional use of other cytokines, culture conditions, the cocktail of reagents utilized for maturation and the methods of antigen loading vary considerably, it is reasonable that the quality and biological activity of each DC preparation have been shown to differ [3, 18, 22-26].

In an attempt to simplify the methods currently being used for DC generation, we found that enrichment of monocytes as an initial step was not essential since functional DCs were successfully generated from unfractionated PBMCs. During the process of optimizing such a protocol for the eventual use for immunization of HIV-1 infected patients, we found that an exposure of PBMCs to inactivated HIV-1 (iHIV-1) at the initiation of culture resulted in a marked reduction in the yield of DCs due to cell death. Studies designed to elucidate the mechanisms for such cell death revealed that IFN-α produced from the iHIV-1-exposed $CD123^+$ plasmacytoid DCs in PBMCs impairs DC generation *in vitro*. Results of these studies constitute the basis of this report.

2. Subject and Methods

2.1. Reagents

Medium used throughout this study consisted of RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum (FCS) (Sigma chemical co, St. Louis, MO), (heretofore referred to as RPMI medium). The recombinant human cytokines used included IL-4, GM-CSF, TNF-α, TNF-β, IL-1β (PeproTech, London, United Kingdom), IFN-α A (α 2a) (R&D System, Minneapolis, MN), IFN-β (Torey, Tokyo Japan), and IL-2 (provided by the U.S. National Institutes of Health Acquired Immune Deficiency Syndrome (AIDS) Research and Reference Reagent Program). Poly I:C was purchased from Alexis Biochemicals (San Diego, CA). Aldrithiol-2 (AT-2) and low-endotoxin bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). The monoclonal antibodies against human cell surface molecules used included anti-CD4 blocking antibody (SIM-2) (from the AIDS Research and Reference Reagent Program), anti-TRAIL (CD253), anti-Fas-L (CD178), anti-CD279 (PD-1) (BioLegend, San Diego, CA), anti-type-I IFN receptor 2 blocking antibody (IFNAR2) (LIFESPAN biosciences, Seattle, WA), anti-TNF-α, anti-TNF-β and anti-TNF receptor 1 (R&D systems, Minneapolis, MN). Fluorescent-dye labeled mouse mAbs used included anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD80, anti-HLA-DR, and isotype-matched control mAbs (Beckman Coulter, Fullerton CA), and anti-CD11c, anti-CD86 and anti-CD83 (BioLegend, San Diego, CA).
ELISA kits for the quantitation of human IFN-α were purchased from MABTECH (Mariemont, OH), human IFN-β from Invitrogen (Carlsbad, CA), human IFN-γ, human IL-10 and IL-4 from Biolegend (San Diego, CA). The human monocyte negative isolation kits, the human T cell isolation kits, and the human naïve $CD4^+$ T cell isolation kits were purchased from Invitrogen (Carlsbad, CA) and Miltenyi Biotec (Gladbach, Germany), respectively. The Vybrant^{TM} CFSE cell tracer kit was purchased from Invitrogen.

2.2. Generation of DC

PBMCs were isolated from heparinized peripheral blood obtained from normal healthy adult volunteer donors by standard density gradient centrifugation. Cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% low-endotoxin BSA and 2 mM Na_2-EDTA. For select experiments, monocytes were purified from these PBMCs by using the $CD14^+$ monocyte negative isolation kit (Invitrogen, Carlsbad, CA). An aliquot of cells from each monocyte preparation was examined by flow cytometry and found to contain >90% $CD14^+$ cells. PBMC (2.5 x 10^6 cells/ml) or the purified monocytes (5×10^5 cells/ml) were cultured in RPMI medium containing human GM-CSF (500 ng/ml) and human IL-4 (200 ng/ml) at 37° C in 24-well plates in a 5% CO_2 humidified incubator for 5 days. In some experiments, these DCs were further matured by incubation with poly I:C (20 ug/ml) and IL-1β (10 ng/ml) for an additional 2 days. The viability of myeloid DCs in each PBMC culture on day 5 was calculated as follows: (the number of viable cells counted by using a Bilker-Chulk hemocytometer) x (percent $CD11c^+$ cells within the viable cell gate as determined by flow cytometry).

2.3. HIV-1 preparation and inactivation

HIV-1_{JR-CSF} viral stock was produced by transfection of the 293T cells with the appropriate HIV-1 infectious plasmid DNA utilizing the calcium phosphate method followed by *in vitro* culture of the transfectd cells in RPMI medium for 2 or 3 days [5]. HIV-1_{IIIB} was cultured and harvested from Molt-4/IIIB cell cultures. Batches of each HIV-1 preparation were inactivated with AT-2 as described previously [5]. These AT-2-inactivated HIV-1 (iHIV) were then purified by pelleting down the virus at 20,000 x g for 2 hr 3 times in 0.1% BSA-PBS. The virus pellet was then resuspended in 0.1% BSA-PBS, aliquoted and stored at -80°C until use. The concentration of HIV-1 was estimated by measuring levels of p24 antigen utilizing an ELISA kit (ZeptoMetrix, Buffalo, NY). An aliquot of 1 μg/ml of the AT-2-treated HIV-1 preparation when incubated with previously activated human PBMCs as previously described [5] failed to demonstrate the presence of any detectable infectious virions.

2.4. Flow cytometry

Aliquots of the cells to be analyzed were incubated in PBS containing 0.1% BSA and 0.1% NaN_3 (FACS buffer) supplemented with 2 mg/ml normal human IgG on ice for 15 min to block Fc receptors. The cell suspension was then incubated with a pre-determined optimal concentration of the appropriate fluorescent dye-labeled mAbs against human cell surface molecules on ice for 30 min. After washing with FACS buffer, cells were fixed in 1%

paraformaldehyde (PFA)-containing FACS buffer. The cells were analyzed by standard flow cytometry using a FACS-Calibur and the data obtained analyzed using the Cell Quest software (BD Pharmingen, San Diego, CA).

2.5. Stimulation of T cells

Enriched populations of naïve $CD4^+$ T cells and bulk T cells with > 90% purity were isolated from normal human PBMCs by using appropriate negative cell isolation kits. These cells were labeled with CFSE (carboxy-fluoresecein diacetate, succinimidyl ester) according to the manufacture's protocol (Invitrogen, Carlsbad, CA). These T cells (4×10^4 cells/well) were co-cultured with allogeneic DCs at a T:DC ratio of 50:1 in 100 ul of RPMI medium supplemented with 20 U/ml human IL-2 in 96-well U-bottom plates. Cell proliferation and cytokine production were determined on day 4.

2.6. Statistical analysis

Data were tested for significance using the Student's *t* test by using Prism software (GraphPad Software).

Results

3.1. Myeloid DCs are generated from bulk PBMC cultures in the presence of IL-4 and GM-CSF.

According to the most commonly used current protocols to generate myeloid DCs *in vitro*, the precursor monocytes are first commonly enriched from PBMCs with varying degrees of efficiency by a variety of methods prior to cultivation. In an attempt to reduce the cost, the labor and any loss of potential precursors from PBMCs, we first attempted to determine whether functional DCs could be generated by culturing bulk unfractionated PBMC's in the presence of IL-4 and GM-CSF for 5 days followed by a 2-day maturation by incubation in media containing poly I:C and IL-1β. As shown in Fig.1a, cells expressing high levels of CD11c, CD83, HLA-DR and CD86, highly reminiscent of mature myeloid DCs, represented 10% of the cultured cells. The other smaller cells (90% in the viable fraction) consisted of 90% $CD3^+$ T and 10% $CD20^+$ B cells. The average yield of DCs from 2.5×10^6 PBMC cultures was $2 \sim 2.5 \times 10^5$ cells depending on the donor.

The PBMC-derived myeloid DCs (PB-DCs) showed potent allo-T cell stimulating activity at levels comparable to those generated from purified monocytes (Mo-DCs) (Fig.1b). In separate experiments, similarly generated ovalbumin (OVA)-pulsed PB-DCs were also able to induce OVA-specific T cell and OVA-specific antibody responses using the hu-PBL-SCID mouse model (Kodama et al. manuscript in preparation). Thus, it is clear that functional immuno-stimulating DCs can be generated from bulk PBMCs without the prior requirement for using enriched population of monocytes *in vitro*.

3.2. AT-2-inactivated HIV-1 (iHIV) impairs generation of DCs from PBMCs.

Previously, our laboratory reported that conventionally prepared DCs sensitized with iHIV-1 induce HIV-1 specific Th1 immune responses in the hu-PBL-SCID mice [6]. In efforts to test whether the bulk PB-DCs could similarly induce such anti-HIV-1 T cell responses in the hu-PBL-SCID mice, attempts were made to first determine the time of culture and dose of iHIV-1 which was optimal for the derivation of sensitized PB-DCs. To our surprise, incubation of the PBMC cultures with such iHIV-1 at the initiation of the culture, resulted in impairment of DC development by day 5. However, such impairment of DC development was not observed in the iHIV-1 pulsed purified monocyte cultures set up in parallel (Fig.2a). In addition, if the iHIV-1 was added to the cultures on day 3, such impairment was not detectable on day 5 (data not shown).

As shown in Fig. 2b, flow cytometric analysis of the cells cultured for 5 days in the presence of iHIV-1 showed a marked dose-dependent impairment in the generation of DCs as determined by examining the high forward and side scatter profile of an aliquot of such cultured cells (typically used for the analysis of DCs). Such impairment by iHIV-1 exposure appeared to be induced as early as day 3 post culture as determined by flow cytometry (Fig.2c). It was reasoned that apoptosis of the cells could account for such impairment and thus aliquots of similar cultures were analyzed for the frequency of Annexin-V binding cells. As seen in Fig.2d, there were more Annexin-V-binding apoptotic cells in the iHIV-1 exposed PBMCs as early as 24 hrs post culture as compared to the control non-iHIV-1 exposed cultured cells ($P<0.01$). Taken together, these results show that iHIV-1 impaired the generation of DCs from unfractionated PBMCs in a dose-dependent fashion, which is secondary to apoptosis. The impaired generation of

DCs was also not secondary to residual levels of AT-2 since the use of mock virus preparation treated with AT-2 had no detectable effect on such cultures (data not shown).

3.3. Both CCR5- and CXCR4-tropic using HIV-1 isolates impair DC generation from PBMCs and modify CD86 expression.

The role of co-receptor tropism of HIV-1 isolates in the impairment of DC generation was examined next. As shown in Fig.3a, both the CCR5-tropic HIV-$1_{JR\text{-}CSF}$ and CXCR4-tropic HIV-1_{IIIB} isolates that had been inactivated with AT-2 similarly reduced the numbers of viable myeloid DCs generated from unfractionated PBMCs on day 5. Again, no impairment was observed in the similarly iHIV-1-exposed purified monocyte cultures by either of the iHIV-1 isolates. It was of interest to note that the DCs that remained in the iHIV-1-exposed PBMC cultures expressed about 6-8-fold higher levels of CD86 (note the MFI scale) on their cell surface than the DCs from the control non-HIV exposed ones even though they were not stimulated for maturation (Fig.3b). However, these high density $CD86^+$ HIV-1-exposed DCs led to reduced levels (approx. 50%, $p< 0.05$) of IFN-γ production when co-cultured with allogeneic T cells as compared with the DCs from the control cultures (Fig.4). In contrast, iHIV-1 treated and untreated Mo-DCs induced similar levels of IFN- γ when co-cultured with allogeneic T cells (Fig.4). The decreased levels of IFN-γ were not secondary to increase in the levels of IL-4 or IL-10 production. These data indicate that the *in vitro* impairment of DC generation from unfractionated PBMCs is independent of HIV-1 co-receptor usage and the residual DCs show markedly reduced capacity to induce IFN-γ although they expressed high levels of CD86.

3.4. IFN-α is involved in the impairment of DC generation

The previous published reports that type-I IFNs inhibit DC generation *in vitro* [27, 28] prompted us to determine whether the synthesis of type-I IFNs was the basis of iHIV-1-mediated impairment of DC generation. As shown in Fig.5a, the addition of either recombinant IFN-α or the type-I IFN-inducing agent poly I:C could induce impairment of DC generation similar to that induced by iHIV-1 in DC from not only unfractionated PBMCs but also from highly enriched preparation of monocytes. Again, the DCs that remain in these IFN-α or poly I:C-treated cultures expressed high levels of CD86 (Fig.5a). These IFN-α or Poly I:C exposed MO-DCs showed diminished function as measured by their ability to induce allogeneic Th1 responses (data not shown) similar to that noted for iHIV-1 exposed PB-DCs. Importantly, the impaired generation of DCs induced by iHIV-1, IFN-α and poly I:C were specifically reversed by the prior addition of anti-type-I IFN-R antibody to the cultures (Fig.5b). Altogether, these data suggest that type-I IFNs produced by unfractionated PBMC in response to iHIV-1 is the basis for the impaired generation of DC *in vitro.*

The levels of both IFN-α and β in the supernatant fluid of unfractionated PBMCs cultured in the presence of iHIV-1, poly I:C stimulated unfractionated PBMCs and monocytes were also quantitated by ELISA. As seen in Fig.6a, high levels of IFN-α but not IFN-β were measured in such supernatant fluids, suggesting that it is IFN-α not β that is the cytokine that is likely involved. Since pDCs are known to be a major IFN-α producing cell lineage, we next tested whether pDCs were involved in our culture system. Freshly obtained PBMCs were thus depleted of pDCs by incubation with anti-CD123 antibody-conjugated magnetic beads and the remaining PBMCs were cultured in the presence of iHIV-1 using our standard culture system. As shown in Fig.6b, prior depletion of CD123-bearing pDCs from PBMCs markedly decreased the ability of iHIV-1 to impair the generation of DC's *in vitro*. Taken together, these data suggest that IFN-α produced by pDCs in response to iHIV-1 is involved in the diminished generation of DCs from unfractionated PBMCs *in vitro.*

3.5. HIV-1 impairs DC generation from PBMCs in a CD4 dependent fashion.

Since an interaction between HIV-1 gp120 and CD4 has been shown to be requisite for IFN-**α** production by pDCs, we next examined the effect of blocking anti-CD4 antibody on DC generation from PBMCs. As shown in Fig.7, the prior addition of anti-CD4 antibody ameliorated the inhibitory effect of iHIV-1 on DC generation (Fig.7a) and CD86 up-regulation (Fig.7b), and inhibited the production of IFN-α (Fig.7c) in the iHIV-1-exposed PBMCs, confirming that IFN-α is produced by pDCs in a CD4 dependent fashion.

3.6. TNF blocks the DC impairment by AT-2 inactivated iHIV-1.

Finally, we examined whether the common apoptosis inducing cytokines TNF-α, TNF-β and the apoptosis-related cell surface antigens including FasL, TRAIL and TNF were involved in the impairment of DC generation from iHIV-1-exposed PBMCs. As shown in Fig.8a, the addition of blocking antibodies against FasL, TRAIL, TNF-α and TNF-β and TNF-R, failed to show any detectable effects on the impaired generation of DCs by iHIV-1. This failure was not secondary to the amount of antisera utilized. To our surprise, however, the addition of soluble recombinant TNF-α (or TNF-β) to the iHIV-1-exposed PBMC cultures at the time of initiation of the cultures reversed the iHIV-1-mediated inhibition of DC generation from PBMCs (Fig.8b). Consistent with these findings was the observation that the addition of TNF-α to these cultures led to a marked reduction in the level of IFN-α production from these PBMCs (Fig.8c). Therefore, these data suggest that iHIV-1 induced IFN-α directly impairs DC generation, and that TNF counteracts the iHIV-1 effect by reducing IFN-α production by pDCs in PBMCs.

4. Discussion

Data presented herein show that myeloid DCs can be generated *in vitro* by culturing unfractionated human PBMCs in media containing IL-4 and GM-CSF for 5 days followed by 2 days of further maturation in media containing poly I:C and IL-1β. These PB-DCs expressed high levels of CD86 and HLA-DR, and were capable of inducing not only alloreactive T cell responses *in vitro* but also antigen-specific T and B cell responses *in vivo*. This finding is important to reduce the cost, the labor and any loss of precursors from PBMCs in generating immuno-stimulating DCs *in vitro*. The superiority of using unfractionated PBMCs over enriched population of monocytes for the generation of immature DCs with high rates of recovery has also been reported by Goxe et al. [29]. They showed that unfractionated PBMCs cultured in a serum-free medium containing IL-13 and GM-CSF led to 38~54% higher recovery than the use of enriched population of monocytes. However, in the studies reported herein, we observed little or no difference in the yields of DCs between PBMCs and highly purified monocyte cultures. This discrepancy is likely due to either the higher efficiency of the kit we utilized for the isolation of monocytes or differences in the cytokines used for the derivation of the DCs which included IL-4 instead of IL-13 in our studies. The fact that one can utilize unfractionated PBMCs for the generation of highly functional DCs *in vitro* reduces not only the time involved in setting up cultures but also limits the number of manipulations and facilitates the generation of such cultures for clinical studies. Thus, the present data may provide an alternative simple and low-cost protocol for the *in vitro* generation of conventional human DCs with immuno-stimulating function. The role of the residual population of lymphoid cells in such cultures remains to be elucidated. The fact that there was no detectable expression of CD69 on such residual lymphoid cells (data not shown) supports the view that there is minimal if any effect of such quiescent cells on the generation of DC's. Further studies on this issue are in progress.

The finding that both CCR5- and CXCR4-tropic iHIV-1 interfered with DC generation from unfractionated PBMCs when added at the initiation of culture is important because of its potential *in vivo* relevance. It is known that 60-70% of monocytes are prone to die of apoptosis and necrosis by 7 days when left unstimulated *in vitro*, with the main loss occurring within the first 24 hrs [30, 31]. Such spontaneous monocyte apoptosis can be ameliorated to varying degrees by the addition of a variety of pro-inflammatory cytokines [32] or agents such as LPS [33]. It has also been reported that even in the presence of both IL-4 and GM-CSF about 40% of monocytes spontaneously undergo apoptosis during maturation into DCs *in vitro* [34]. The results of the studies reported herein show that approximately 50% of the monocytes (or DCs) from the PBMCs die following exposure for 5 days to iHIV-1. Since IFN-α alone could induce a similar loss of DCs in both unfractionated PBMCs and monocyte cultures, and that anti-type-I IFN receptor antibody reversed the effects of both iHIV-1 and IFN-α, it is clear that IFN-α is likely the major effector molecule responsible for the impairment of DC generation induced by iHIV-1. Similar impairing effects of type-I IFNs on monocyte-derived DC has been previously reported by McRae [28]. In contrast, Lehner et al. [27] reported that IFN-α alone is not capable of inducing impairment of DC generation unless an additional stimulus like LPS or LTA was included in such cultures. Since the media and cytokines that we utilized for the studies reported herein were free of endotoxin and bacterial contamination, the reason for this discrepancy remains undefined. Based on the results of our studies, the major mechanism for the decrease in the yield of viable DCs by iHIV-1 appear to be cell death, and was not likely due to static suppression of differentiation. Recently, it has been demonstrated that IFN-β induces apoptosis of immature murine DCs thorough caspase activation [35]. Therefore, it is possible that human IFN-α similarly induces apoptosis of immature DCs directly through type-I IFN receptors. Since soluble gp120 has been previously shown to induce $CD4^{+}$ T cell apoptosis, studies were conducted to determine whether soluble gp120 (commercially available) could inhibit conventional DC generation from PBMCs and monocytes in our culture system. Results of these studies showed that the addition of soluble gp120 to such cultures did not have

any detectable inhibitory effect (data not shown), indicating that whole HIV-1 virion is required for the induction of monocyte apoptosis.

Results from a large number of studies have documented the role of type-I IFNs in the modulation of DC biology at different levels. In addition to its well-known antiviral effect, type-I IFNs induce the maturation of DCs [5, 36-38]. However, it has been shown that when fresh monocytes are cultured in the presence of IFN-α or IFN-β at culture initiation, their maturation into DCs and IL-12 producing activity is severely diminished [28, 39]. It should be noted that in the culture system utilized in the studies reported herein some (about 50%) DCs survived in the iHIV-1 or IFN-α treated PBMCs. It is possible that these remaining DCs are resistant to the effects of IFN-α because they are a fraction of the terminally differentiated DCs that are known not to express receptors for IFN-α [40]. On the other hand, these cells could have developed resistance to undergo apoptosis. The fact that these remaining DCs as compared with control DCs were poor inducers of Th1 responses despite their expression of high levels of CD86 similar to previous observations [28, 39] suggests that either these cells are immune exhausted or that such culture conditions lead to the down regulation of some other co-stimulatory molecules required for optimum Th1 cell activation.

Based on the present data, it is clear that IFN-α secreted from $CD123^+$ pDCs as a result of interaction between HIV-1 gp120 and CD4 affects DC maturation, which is in support of previous findings [41, 42]. The reasons why iHIV-1 can induce large amounts of IFN-α has been thought to be due to the high efficiency by which CD4 expressing pDCs pick up HIV-1 particles through the interaction with envelope gp120 followed by pinocytosis of HIV-1 into the endosome compartments where viral RNA stimulates TLR-7, which results in the production of IFN-α [43-45]. The HIV-1-induced IFN-α has also been reported to induce bystander maturation of myeloid DCs [46]. Whereas the impairment of DC generation by iHIV-1 was found to be CD4 dependent, the addition of anti-CD4 to such cultures did not completely block IFN-α production (Fig.7c). While the reasons for the failure of anti-CD4 to completely block IFN-α production remains to be defined, we reason that some whole virion or degraded components of HIV-1 can be incorporated into pDCs in a CD4-independent manner and stimulate IFN-α production.

The present data also showed that FasL, TRAIL, TNF-α and TNF-β have limited if any relevance to the impairment of DC generation from PBMCs induced by iHIV-1, IFN-α or poly I:C. Although the major mechanism responsible for cell death still remains to be elucidated, it is of interest that TNF-α or TNF-β reversed the iHIV-1-, IFN-α-, or poly I:C-mediated DC impairment. TNF has been known to act not only in maturation [47] and killing of DCs [48], but also in the prevention of apoptosis of monocytes [32]. Recently, it has been reported that TNF-α blockade impairs DC survival and function in rheumatoid arthritis [49], suggesting that TNF may play a role in determining the longevity of DCs. Our present data suggest that the blocking activity of TNF against iHIV-1 or type-I IFN-mediated impairment of DC generation may be related to the down-modulation of IFN-α production from pDCs, since TNF did not act to down-modulate type-I IFN receptors expressed on monocytes (data not shown). Alternatively, there may be additional mechanisms by which TNF rescues DC generation in the presence of iHIV-1. Further studies are in progress aimed at analysis of the biological role of TNF in DC generation under inflammatory conditions.

In conclusion, data from the present study suggests that unfractionated PBMCs are a good source for the generation of conventional DCs *in vitro* in media supplemented with IL-4 and GM-CSF, and that HIV-1 impairs DC differentiation from PBMCs due to apoptosis via pDC-produced IFN-**α**. Therefore, strategies need to be devised to inhibit and/or limit the production of type-I IFNs in such cultures in order to maintain a good yield of functional DCs, especially from PBMC samples from individuals infected with HIV-1.

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