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Host chemokine (C-C motif) ligand-2 (CCL2) is differentially regulated in HIV type 1 (HIV-1)-infected individuals

Abdul Wahid Ansari[1,2], **Nupur Bhatnagar**[2], **Oliver Dittrich-Breiholz**[3], **Michael Kracht**[3], **Reinhold E. Schmidt**[2] and **Hans Heiken**[2]

[1]MD/PhD Program, [2]Department of Clinical Immunology and [3]Institute of Molecular Pharmacology, Hannover Medical School, Carl-Neuberg-Street 1, D-30625 Hannover, Germany

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Abstract

Several cytokines and chemokines including chemokine (C-C motif) ligand-2 (CCL2) are induced in HIV-1 infection. However, the impact of HIV-1 viremia on CCL2 regulation is largely unknown. We utilized a DNA oligonucleotide microarray covering 110 inflammatory genes. Five genes were induced by at least 2-fold in PBMCs of HIV-1 viremic (>100.000 RNA copies ml^{-1}) as compared with aviremic (<50 RNA copies ml^{-1}) individuals. These genes were CCL2, CXC chemokine ligand-10, IFN-γ, GTP-cyclohydrolase-1 and C-C chemokine receptor-1. In addition to microarray data verification by real-time PCR, analysis of independent patient samples revealed a similar expression pattern. CCL2 was the most strongly regulated gene at mRNA level and its serum concentration was significantly elevated in viremic compared with aviremic and HIV-1 seronegative controls, indicating a positive correlation between viremia and CCL2. Flow cytometric studies demonstrated a higher percentage of CCL2-expressing CD14+ monocytes in viremic compared with aviremic individuals. These results suggest a highly restricted modulation of host inflammatory gene response by HIV. Genes up-regulated in the viremic state, in particular CCL2, presumably serve as potential enhancing factors in HIV-1 replication, represented by high viral load in HIV-1 viremic patients. Inhibition of increased CCL2 production could provide a new therapeutic intervention in HIV-1 infection.

Introduction

HIV type 1 (HIV-1) infection transforms the host's cellular environment into a favorable niche for its replication and survival (1, 2). In most instances, an alteration of the cytokine and chemokine network is observed. However, potential effects of viremia on these inflammatory mediators are not fully elucidated. Monocytes and macrophages are not only the prime targets for HIV-1 infection but also a source of inflammatory cytokines and chemokines (3). These key modulators of the immune system play a critical role in recruiting new target cells including CD4+ T cells to the site of infection (4). In the past few years, significant progress has been made to understand the molecular mechanisms underlying the host–pathogen interaction with the advent of microarray technology (5, 6). However, until now we do not know the crucial host factors influencing this host–virus relationship. There are reports about general impact of HIV-1 on host cell gene expression both in patient-derived B and CD4+ T cells as well as in *in vitro* infected cells (7–10). However, the role of HIV-1 viremia on host cell cytokine/chemokine expression has not been studied in detail.

During HIV-1 infection, a series of cytokines and chemokines is induced which may either support or inhibit viral replication. For example, the C-C chemokines macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4 and regulated upon activation, normal T cell expressed and secreted (RANTES)/CCL5 inhibit M-tropic HIV-1 infection by competing with the virus for its binding to the co-receptor CCR5 (11–17). In contrast, there are reports demonstrating stimulation of HIV-1 replication by the chemokines growth-regulated oncogene-α, CXCL10 and IFN-γ inducible protein (IP)-10/CXCL10 (18, 19).

Macrophage chemoattractant protein (MCP)-1, also known as CCL2, attracts monocytes, T lymphocytes and NK cells *in vitro* (20, 21). CCL2 is expressed by various cell types and is induced after HIV-1 infection of macrophages (22). CCL2

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Correspondence to: H. Heiken; E-mail: heiken.hans@mh-hannover.de and A. W. Ansari; E-mail: ansari.wahid@mh-hannover.de

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along with MIP-1α, MIP-1β and RANTES has been demonstrated to increase the replication of T-tropic strains in CD4+ T cells (23). Recently, it has been reported that CCL2 plays a critical role in the neuropathogenesis of HIV-1-associated dementia and protects human neurons and astrocytes from *N*-methyl-D-aspartate or HIV-tat-induced apoptosis (24).

To investigate the impact of HIV-1 viremia on the host inflammatory cytokine/chemokine network, more specifically on CCL2, we utilized DNA microarray approach on PBMC RNA derived from HIV-1-infected viremic and aviremic individuals. In this study, we report that HIV-1 viremic patients show an altered expression of key inflammatory cytokines and chemokines as compared with aviremic individuals. In particular, CCL2 is highly regulated both at mRNA and protein level, demonstrating a correlation between viremia and host CCL2 response.

Methods

Patients and control subjects

A total of 10 aviremic and 10 viremic HIV-1 patients were analyzed for gene expression studies. For determination of serum CCL2 concentration, 36 HIV-1 patients were selected based on their viral load (18 aviremic and 18 viremic). Clinical characteristics and therapy status of each patient are given in Table 1. In addition to above subjects, a new cohort of patients (n = 16) was recruited for CCL2, CXCL10 and IFN-γ gene expression analysis by real-time PCR. For plasma viral load measurement, peripheral blood was drawn in EDTA-coated tubes and stored at –80°C until use. Viral load was detected by standard or ultra-sensitive COBAS Amplicor HIV-1 Monitor™ test version 1.5 (Roche Diagnostics, Mannheim) or by b-DNA analyzer System 340 (Bayer Diagnostics, Fernwald) at our center. All subjects except patient number A6, B7, B9 and C13 were on highly active anti-retroviral therapy (HAART) at the time of this study. Written informed consent from each subject and approval from Institutional Ethics Review Board were obtained before this study.

PBMC isolation and RNA extraction

Heparinized venous blood was drawn from HIV-1-infected viremic individuals maintaining a viral load of >100 000 RNA copies ml^{-1} and from aviremic individuals with undetectable viral load of <50 RNA copies ml^{-1} in their plasma. PBMCs were isolated from these patients by conventional Ficoll–Hypaque (Biochrome AG, Berlin) gradient centrifugation. Cells were washed with PBS and RNA extraction procedure was immediately followed according to manufacturer's protocol of RNeasy Mini kit (Qiagen, Hilden, NL, USA). All RNA samples were treated with DNAseI (RNase-free DNase set, Qiagen) to eliminate genomic DNA contamination.

Oligonucleotide DNA microarray experiments

The microarray used in this study was the first version of the inflammation array (MWG Biotech) and contained 110 oligonucleotide probes for inflammatory genes that were previously validated by our laboratory (25) as well as five 'housekeeping' genes. Each probe was spotted twice in random order. Total RNA from PBMC of aviremic and viremic patients as indicated in the legend of Table 2 was purified with a Qiagen RNeasy kit followed by 'on column' DNaseI digestion (Qiagen). RNA was used to prepare Cy3-labeled cRNA by oligo dT-T7-primed double-stranded cDNA synthesis (cDNA synthesis system, Roche) followed by *in vitro* transcription with T7-polymerase (MEGAscript T7 kit, Ambion) as directed by the manufacturers. cDNA yield was determined photometrically. Equal amounts of cRNAs derived from ~2.5 μg of total RNA were hybridized individually to microarrays in pre-prepared hybridization solution (MWG Biotech) at 42°C overnight and then washed sequentially in 2× SSC, 0.1%

Table 1. Clinical features of HIV-1-infected individuals

Patient	CDC category	Status	Viral load (copies ml^{-1})	CD4+ T cells μl^{-1}	CD8+ T cells μl^{-1}	CD4/CD8 ratio	Therapy[a]
A1	II (A3)	Aviremic	<50	282	740	0.38	HAART
A2	II (B2)	Aviremic	<50	917	1132	0.81	HAART
A3	I (A2)	Aviremic	<50	852	1353	0.63	HAART
A4	III (C3)	Viremic	>750.000	6	352	0.02	HAART
A5	III (C3)	Viremic	>750.000	4	157	0.02	HAART
A6	III (C3)	Viremic	>750.000	72	245	0.30	None
B1	III (B3)	Aviremic	<50	563	1018	0.55	HAART
B2	III (C2)	Aviremic	<50	522	562	0.93	HAART
B3	III (C3)	Aviremic	<50	194	972	0.20	HAART
B4	I (A2)	Aviremic	<50	893	913	0.98	HAART
B5	III (C3)	Aviremic	<50	411	1191	0.35	HAART
B6	II (A3)	Viremic	419 000	330	2475	0.13	HAART
B7	III (B3)	Viremic	>750.000	168	641	0.26	None
B8	III (C3)	Viremic	>750.000	31	2585	0.01	HAART
B9	III (C3)	Viremic	>750.000	298	1649	0.18	None
B10	III (C)	Viremic	>750.000	6	205	0.03	HAART
C11	I (A2)	Aviremic	<50	983	564	1.74	HAART
C12	III (C3)	Aviremic	<50	467	701	0.67	HAART
C13	II (A3)	Viremic	>500.000	22	429	0.05	None
C14	III (C3)	Viremic	118.897	11	633	0.02	HAART

HIV-1-infected patients were categorized into two groups: either aviremic with <50 copies RNA ml^{-1} or viremic with >100.000 copies RNA ml^{-1}.
[a]HAART included at least one protease and/or one non-nucleoside reverse transcriptase inhibitor with two reverse transcriptase inhibitors of HIV-1.

Table 2. Identification of differentially expressed genes comparing aviremic with viremic HIV-1 patients by microarray analysis

Oligo name	RefSeq accession	RefSeq gene name	Experiment 1			Experiment 2			Experiment 3				
Human inflammation array			Fluorescence intensity		Ratio viremic/aviremic	Fluorescence intensity		Ratio viremic/aviremic	Fluorescence intensity				Ratio viremic (mean)/aviremic (mean)
			Aviremic	Viremic		Aviremic	Viremic		Aviremic	Aviremic	Viremic	Viremic	
			$n = 3$ (A1–A3)	$n = 3$ (A4–A6)		$n = 5$ (B1–B5)	$n = 5$ (B6–B10)		(C11)	(C12)	(C13)	(C14)	
mwginflhum#008	NM_002982	*CCL2*	90	666	7.4	27	518	19.3	31	29	140	320	7.7
mwginflhum#009	NM_001565	*CXCL10*	164	328	2.0	31	186	6.0	28	53	340	185	6.5
mwginflhum#074	NM_000619	*IFN-γ*	227	1090	4.8	75	310	4.2	27	95	661	128	6.5
mwginflhum#056	NM_000161	*GCH1*	171	524	3.1	56	175	3.1	95	106	317	191	2.5
mwginflhum#020	NM_001295	*CCR1*	213	441	2.1	112	254	2.3	47	43	199	182	4.3
mwginflhum#hk01	NM_001101	*actb*	10 718	11 778	1.1	7575	8277	1.1	3475	5997	5835	4110	1.0
mwginflhum#hk02	NM_002046	*gapd*	4567	7984	1.7	2923	2878	1.0	2415	2146	2236	1835	0.9
mwginflhum#hk05	NM_002948	*rpl15*	44 164	51 096	1.2	33 898	22 575	0.7	34 431	21 564	17 082	20 523	0.7
mwginflhum#hk06	NM_021009	*ubc*	50 701	65 264	1.3	59 065	49 994	0.8	28 164	36 710	39 209	30 364	1.1

Two pools of RNA generated from PBMC of three (patients A1–A6, experiment 1) or five (patients B1–B10, experiment 2) or RNA isolated from two individual patients (patients C11–C14, experiment 3), respectively, were used to compare inflammatory gene expression in the aviremic with the viremic state. Microarray experiments were performed as described in Methods. Original spot intensity measurements for the group of genes that was consistently up-regulated by at least 2-fold in PBMC of viremic patients and whose signal intensity was at least 3% of the average signal intensity of all genes on that particular array are shown. For comparison, the spot measurements of four 'housekeeping' genes are also shown. Identifiers for the genes (RefSeq accession number) and for the oligonucleotide probes (oligo name of the MWG human inflammation array) are shown. For data on the individual patients (indicated by capital letters followed by numbers) see Table 1. The complete set of DNA microarray data can be obtained upon request (Kracht.Michael@mh-hannover.de).

SDS, 1× SSC and 0.5× SSC. Hybridized arrays were scanned at maximal resolution on an Affymetrix 428 scanner at six different PMT voltage settings. Fluorescence intensity values from Cy3 channels were processed using Imagene 4.2 software (Biodiscovery). In order to obtain maximal signal intensities without saturation effects, intensity values from six TIFF images were integrated into one value per probe by the MAVI software (Version Pro 2.5.1, MWG Biotech). The mean value from replica spot measurements for each individual gene were used for further analysis using Excel.

Quantitative real-time PCR

Highly regulated and HIV-1 pathogenesis-associated genes were selected for further verification by real-time PCR. Approximately 1–2 μg of pooled RNA from each patient group or individual patients were reverse transcribed using oligo(dT) and Omniscript reverse transcriptase (Qiagen) according to manufacturer's instructions. Diluted cDNA was used as template and amplified using gene-specific primers (Table 3). Primers were designed with the aid of Primer Express Software (PE Applied Biosystems GmbH, Weiterstadt) and synthesized by MWG Biotech. The Taqman β-actin control reagents (PE Applied Biosystems) or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were standard internal housekeeping controls. All amplifications were performed either with ABI Prism 7700 SDS (PE Applied Biosystems) or ICycler (BIORAD, Munich) using 10 μM concentration of each primer in a final reaction volume of 25 μl in duplicates for 40 cycles using SYBR Green I chemistry. To obtain a standard curve, transcribed cDNA was serially diluted. mRNA levels were normalized according to the expression of β-actin/GAPDH in the same sample and values obtained for viremic patients expressed relative to those obtained for aviremic patients which were set as 1. Fold change in the genes were calculated by $2^{-\Delta\Delta C}$ T method (26).

Estimation of CCL2 concentration by ELISA

To determine the level of CCL2 production in HIV-1-infected aviremic and viremic patients, we examined serum of 48 individuals. Eighteen viremic and 18 aviremic HIV-1 patients and 12 HIV-1 seronegative healthy controls were included. All aviremic and majority of the viremic patients were receiving anti-retroviral therapy at the time of study. Sandwich CCL2

Table 3. List of gene-specific primers used in real-time PCR

Genes	Sequences
GAPDH	Forward 5′-GAAGGTGAAGGTCGGAGT
	Reverse 5′-CATGGGTGGAATCATATTGGAA
CCL2	Forward 5′-GTCTCTGCCGCCCTTCTGTG
	Reverse 5′-AGGTGACTGGGCATTGATTG
CXCL10	Forward 5′-CTGAATCCAGAATCGAAGGCC
	Reverse 5′-TG ATCGATTTTGCTCCCC
IFN-γ	Forward 5′-TTGAATGTCCAACGCAAAGC
	Reverse 5′-CGACCTCGAAACAGCATCTGA
GCH1	Forward 5′-AACCATAGCTTCCACGCACCT
	Reverse 5′-CGTTGGTACGATACGCTTTGG
CCR1	Forward 5′-GGCTTCCATGCCAGGCTTATA
	Reverse 5′-CAGAGCCTGAAACAGCTTCCA

ELISA (Pepro Tech, London, UK) with a range of 8–3000 pg ml^{-1} was used for detection. Serum was obtained and stored at –80°C until use. Analysis was done according to manufacturer's protocol with a brief change in use of substrate as ABS (KPL, MD, USA). Assay was done on diluted serum samples in duplicate on a 96-well microtiter plate (Nunc Adsorp) and measured at 450 nm.

Flow cytometry

Frozen PBMC of viremic (n = 4) and aviremic (n = 6) patients were cultured in RPMI 1640 (Biochrom AG, Berlin) complete media. Approximately 1×10^6 cells ml^{-1} were stimulated for 24 h with 50 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Steinheim) at 37°C in a CO_2 incubator in 12-well culture plates (Corning, New York, NY, USA). Brefeldin A 10 μg ml^{-1} (Sigma) was added in the last 4 h of incubation. The following antibodies anti-CD14–FITC (BD Biosciences, Heidelberg), anti-CCL2–allophycoerythrin (APC), anti-CCR2–PE, mouse anti-IgG1–PE and anti-IgG2a–FITC (R&D Systems, Wiesbaden) and anti-IgG2b–APC (Caltag Laboratories GmbH, Brauhaussteg Hamburg) were used for staining.

For surface and intracellular staining, IntraPrep permeabilization reagent (Beckman Coulter, Marseille, France) was applied according to manufacturer's protocol. Phenotypes and cell proportion were analyzed by two-color flow cytometry using FACSCalibur (BD Biosciences, Heidelberg) and CellQuest software. Dead cells were excluded based on forward and side scatter gating.

Statistical analyses

Statistical analyses of two groups of subjects were performed with Student's *t*-test. Evaluation of three groups was performed with one-way analysis of variance test. All analyses were done using Prism 4 (GraphPad) software package. P values <0.05 were considered significant.

Results

Microarray gene expression profile reveals a small number of up-regulated inflammatory genes in HIV-1-infected individuals with high viral load

To understand host cellular inflammatory gene response to HIV-1 infection, we examined a larger range of well-known inflammatory genes in 20 patients, whose clinical features are summarized in Table 1. For microarray screening, four pools of RNAs were generated representing RNA from three and five patients of the aviremic and viremic state, respectively. In addition, RNA samples from four individual patients were also analyzed separately by microarray.

A total of 110 inflammatory genes were analyzed. As indicated in Table 2, five genes were reproducibly up-regulated by at least 2-fold in all experiments. These genes comprised CCL2 (MCP-1), CXCL10 (IP-10), IFN-γ, GCH1 (metabolic enzyme) and CCR1 (MIP-1α receptor). All other genes were either not regulated reproducibly or not expressed at significant levels (data not shown). Furthermore, we could not detect genes that were reproducibly down-regulated in viremic patients.

Differentially expressed genes analyzed by quantitative real-time PCR

mRNA expression of five induced genes detected by DNA microarray was further analyzed by quantitative real-time PCR using gene-specific primers. Analysis of RNA from pooled samples of experiment 2 (according to Table 2) confirmed up-regulation of CCL2, CXCL10, IFN-γ, GCH1 and CCR1 (Fig. 1A). This was also true when re-analyzing RNA from four individual patients shown in Fig. 1(B). Although a certain degree of variability was observed between the two viremic patients, the overall expression of these genes was clearly higher than in both HIV-1 aviremic individuals. Furthermore, increased mRNA transcripts were again detected in CCL2, CXCL10 and IFN-γ when more RNA samples derived from additional aviremic and viremic patients were analyzed individually. Significantly higher mRNA transcript was observed in viremic ($P < 0.05$) compared with aviremic in all genes (Fig. 2A), confirming the consistency with microarray data. CCL2, CXCL10 and IFN-γ showed 3-fold higher expression in viremic patients. However, HAART status in viremic patients did not affect mRNA expression of the analyzed genes.

In addition, *in vitro* HIV-1_{IIIB} infection of healthy donor PBMC resulted in strong induction of CCL2 as measured by real-time PCR (Fig. 2B). These data are in line with previous findings (22, 27). In general, a T-tropic (X4) strain infects CD4 T cells rather than monocytes but there is a small population of CXCR4-expressing monocytes which may become the target cell for X4 viruses, although we cannot formally exclude the possibility of bystander effects mediated by the factors released by CD4 T cell upon HIV-1_{IIIB} engagement. However, there are reports about higher levels of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, IL-6 and IL-1β in HIV-1 disease, and these mediators are also known to play a role in enhancing CCL2 expression. Of note, CCL2 mRNA levels in long-term non-progressors (n = 2) HIV-1 patients were similar to that of aviremic patients (data not shown). Collectively, these findings suggest that a high viremia is associated with a selected set of inflammatory host genes and confers a greater impact on CCL2.

Elevated serum level of CCL2 in HIV-1 viremic patients

To investigate the CCL2 protein level, we performed a CCL2 ELISA on sera from 12 healthy donors, from 18 aviremic patients receiving HAART, and from 18 viremic patients selected on the basis of high viral load who were receiving HAART or not. A significantly higher concentration of CCL2 protein was observed in HIV-1 viremic (P = 0.0008) in comparison with aviremic individuals (Fig. 3A). A positive correlation (r^2 = 0.116 and P = 0.045) between viremia and CCL2 (Fig. 3B) was obtained by linear regression analysis. Regression analysis of CD4/CD8 T cell ratio versus CCL2 pointed to an inverse correlation (P = 0.060 and r^2 = 0.10) which did not reach statistical significance (data not shown). Moreover, additional experiments with intermediate viral load patients ranging between 400 and 100 000 RNA copies ml^{-1} revealed a similar correlation (P = 0.018 and r^2 = 0.122), suggesting that production of CCL2 varies with the viral load (Supplementary Figure 1, available at *International Immunology* Online).

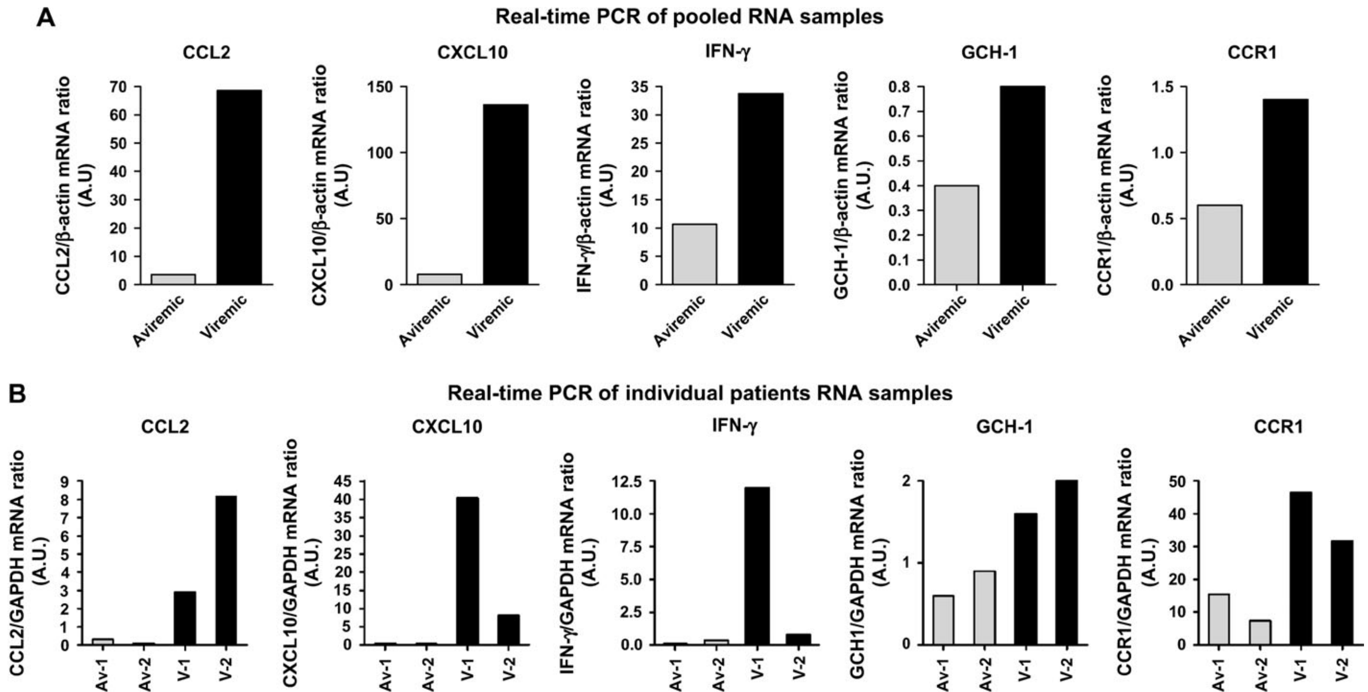

Fig. 1. Quantitative real-time PCR of regulated genes. Pooled PBMC RNAs from HIV-1 viremic and aviremic individuals ($n = 5$) were amplified using gene-specific primers as described in Methods. Expression pattern of up-regulated genes CCL2, CXCL10, IFN-γ, GCH1 and CCR1 are shown in (A). Individual patient's inflammatory gene array verification showed similar expression pattern in two HIV-1 viremic and aviremic individuals (B). Av and V stand for aviremic and viremic individuals, respectively. Fold change in mRNA was quantified in relation to internal housekeeper β-actin/GAPDH mRNA as described in Methods.

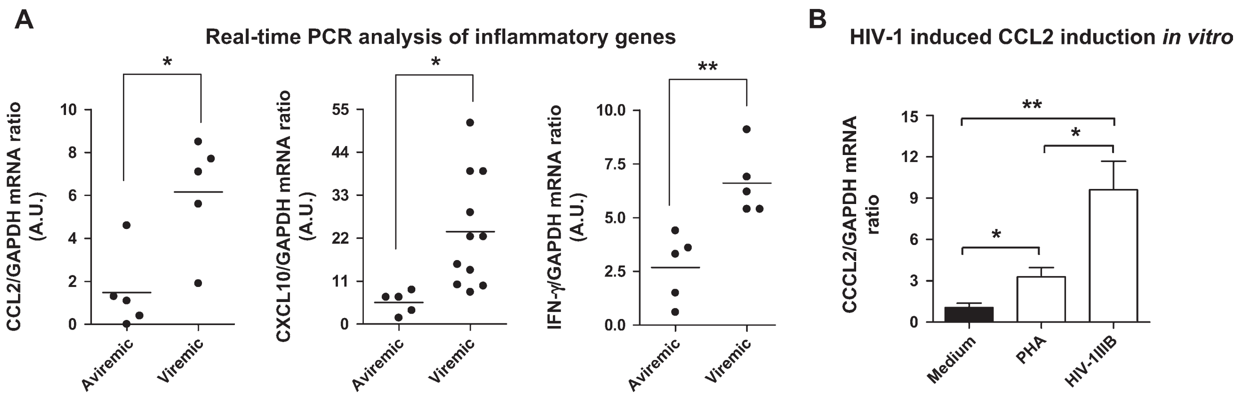

Fig. 2. (A) Transcriptional enhancement of CCL2, CXCL10 and IFN-γ in viremic patients. mRNA expression levels of CCL2, CXCL10 and IFN-γ determined by real-time PCR. Patient's RNA samples were analyzed individually. For CCL2 and IFN-γ expression studies, $n = 5$ each aviremic (<50 copies ml^{-1} and CD4+ T cell count μl^{-1} 245.2 ± 77.4) and viremic (130.665 ± 27.127 copies ml^{-1} and CD4+ T cell count μl^{-1} 322.4 ± 56.2) were recruited. For CXCL10 expression studies, 5 aviremic (<50 copies ml^{-1} and CD4+ T cell count μl^{-1} 245.2 ± 77.49) and 11 viremic (72.869 ± 18.499 copies ml^{-1} and CD4+ T cell count μl^{-1} 263.8 ± 37.6) patient's RNA samples were analyzed. (B) CCL2 gene expression after 24 h incubation with PHA (100 ng ml^{-1}) or T-tropic HIV-1_{IIIB} strain. Briefly, 1×10^6 ml^{-1} healthy donor PBMC ($n = 4$) were incubated with 100 ng ml^{-1} PHA or 50 μl (corresponding to 7.5×10^6 RNA copies ml^{-1}) HIV-1_{IIIB} strain obtained from the Centralized Facility for AIDS Research, NIBSC, UK, was propagated in MT-2 cells. Real-time PCR was performed as described in Methods. Data represented here as mean ± SEM.*$P < 0.05$ and **$P < 0.005$.

Peripheral blood monocytes from viremic patients produce more CCL2 in vitro

Given the fact that viremic patients had elevated concentrations of CCL2 in the serum, it was interesting to know whether the potency of the patient's PBMC to produce CCL2 could be recapitulated *in vitro*. Two-color flow cytometry was performed on previously frozen PBMC samples. In order to obtain uniform conditions, cells were stimulated with PMA for 24 h prior to staining. As expected, monocytes were primary producers of CCL2. The dot plot analysis clearly indicated a higher production of CCL2 by CD14+ monocytes derived from viremic patients (Fig. 4A). There was a highly significant

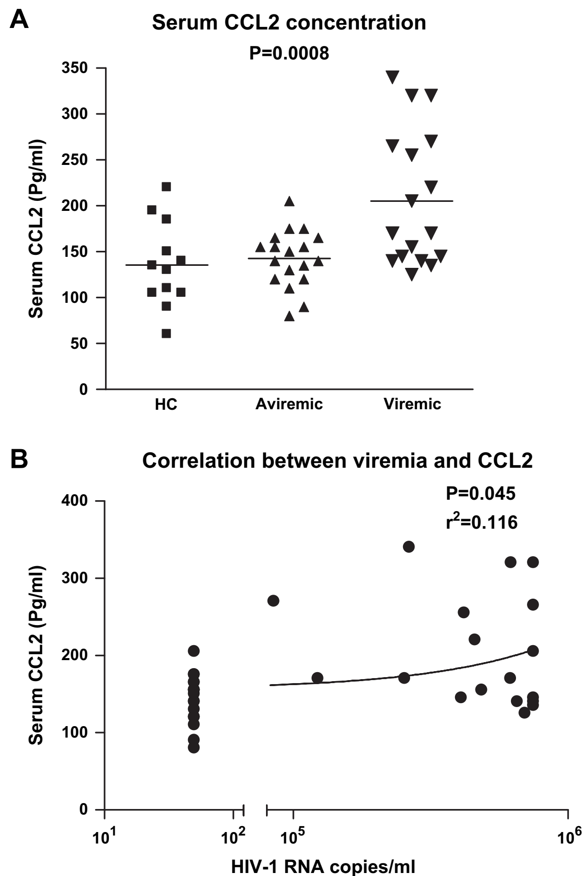

Fig. 3. Detection of CCL2 concentration in the serum of healthy, HIV-1 aviremic and viremic individuals. Effect of viremia on CCL2 production was studied on serum obtained from 18 aviremic (<50 RNA copies, CD4 T cell count 503.4 ± 199.4 with 15 males and 3 females, median age of 47 ± 11.4 years), 18 viremic (viral load 583 944 ± 166 853 CD4 T cell count 210.3 ± 203.3 with 14 males and 4 females, median age of 44.1 ± 8.1 years) and 12 HIV-1 seronegative healthy controls (9 males and 3 females with age 25–35 years). Analysis was performed using sandwich CCL2 ELISA. (A) An elevated level of CCL2 chemokine in HIV-1 viremic (filled inverted triangles) compared with aviremic (filled triangles) and healthy controls (filled squares). (B) Association of HIV-1 viremia with serum CCL2. Statistical analysis was performed with one-way analysis of variance test. Each symbol represents a single individual.

increase in the percentage of CCL2-producing monocytes in viremic ($P < 0.005$) as compared with aviremic patients (Fig. 4B). This was also true when flow cytometry was performed using freshly isolated PMBC of HIV-1 patients (Supplementary Figure 2, available at *International Immunology* Online). Furthermore, we could demonstrate a strong inverse correlation ($P = 0.019$ and $r^2 = 0.515$) between CD4+ T cells and percentage of CCL2+ CD14+ monocytes (Fig. 4C). These findings suggest that viremic patients with low CD4 T cell counts have a high CCL2 expression which could be a potential marker of disease progression. However, we could not observe a significant statistical correlation with viral load (data not shown) except a tendency toward a positive correlation. The data presented here are based on a small cohort of patients ($n = 10$, including six aviremic and four viremic). A possible explanation could be that monocytes are not the sole contributors of CCL2 found in serum, and other cells secrete CCL2 that finally comes into circulation. In contrast to CCL2, we could not detect any significant change ($P = 0.211$) in the expression of the CCR2, the receptor for CCL2, on patient monocytes (Fig. 4D), indicating that viremia had no significant effect on receptor expression.

Discussion

We analyzed 10 aviremic and 10 viremic HIV-1-infected patients by DNA microarray approach. Taking into account that blood samples of the patients had to be collected over a longer period of time and to account for variability among individual patients, RNA samples were divided into three separate microarray experiments in which RNA samples were either pooled or analyzed individually. Furthermore, strict filter criteria were applied to identify the genes that were (i) expressed at measurable levels and (ii) regulated in all experiments by at least 2-fold. This analysis revealed a significant effect of viremia on the mRNA level of CCL2, CXCL10, IFN-γ, GCH1 and CCR1. Induction of CCL2 and CXCL10 are in line with recent studies on SIV- or simian HIV (SHIV)-infected macaques model of HIV encephalopathy (28). To exclude the possibility that the induction of the genes could be the response of one or two patients in the pooled samples, we extended our investigations to a fresh cohort of patients not included in the previous studies using the real-time PCR approach. Again a similar expression pattern in CCL2, CXCL10 and IFN-γ mRNA was observed in viremic as compared with aviremic patients.

These results may imply that persistent infection itself, as present in aviremic patients, does not lead to significant up-regulation of these inflammatory mediators. Only when viral replication is detected, as found in viremic patients, the host inflammatory response leads to an increase in mRNA expression of specific inflammatory genes. Monocytes from HIV-1 patients have been shown to express more CCL2 when compared with healthy controls (29). In this regard, our flow cytometric analysis of patient-derived PBMC showed a clear increase in the CCL2-expressing CD14+ monocytes in viremic patients. This increase in the percentage of CD14+CCL2+ monocytes inversely correlates with the CD4+ T cell count. Since low CD4+ T cell count is associated with viremia and disease progression, an increased expression of CCL2 in these patients could potentially reflect an additional marker of disease progression. CCL2 is induced in many inflammatory diseases including infection with various bacterial and viral pathogens. Production of CCL2 is not specific to HIV-1. Several other viruses have been shown to induce CCL2 expression. For example, viruses such as human rhinovirus (30), respiratory syncytial virus (31), severe acute respiratory syndrome coronavirus (32) and SHIV (33), the best available primate model of HIV-1 infection, have been reported to induce CCL2 expression. Recently, it has been shown that the recruitment of CD4+ T cells by CXCL10 and 11 produced by HIV-1-infected monocyte-derived macrophages and dendritic cells in lymphoid organs contributes to HIV-1 disease (34). In this regard, *in vitro* HIV-1 infection of macrophages is already known to activate CCL2 expression and CCL2 may potentially influence HIV-1 pathogenesis (22). We also observed a similar transcriptional regulation in the CCL2 gene in *in vitro* HIV-1

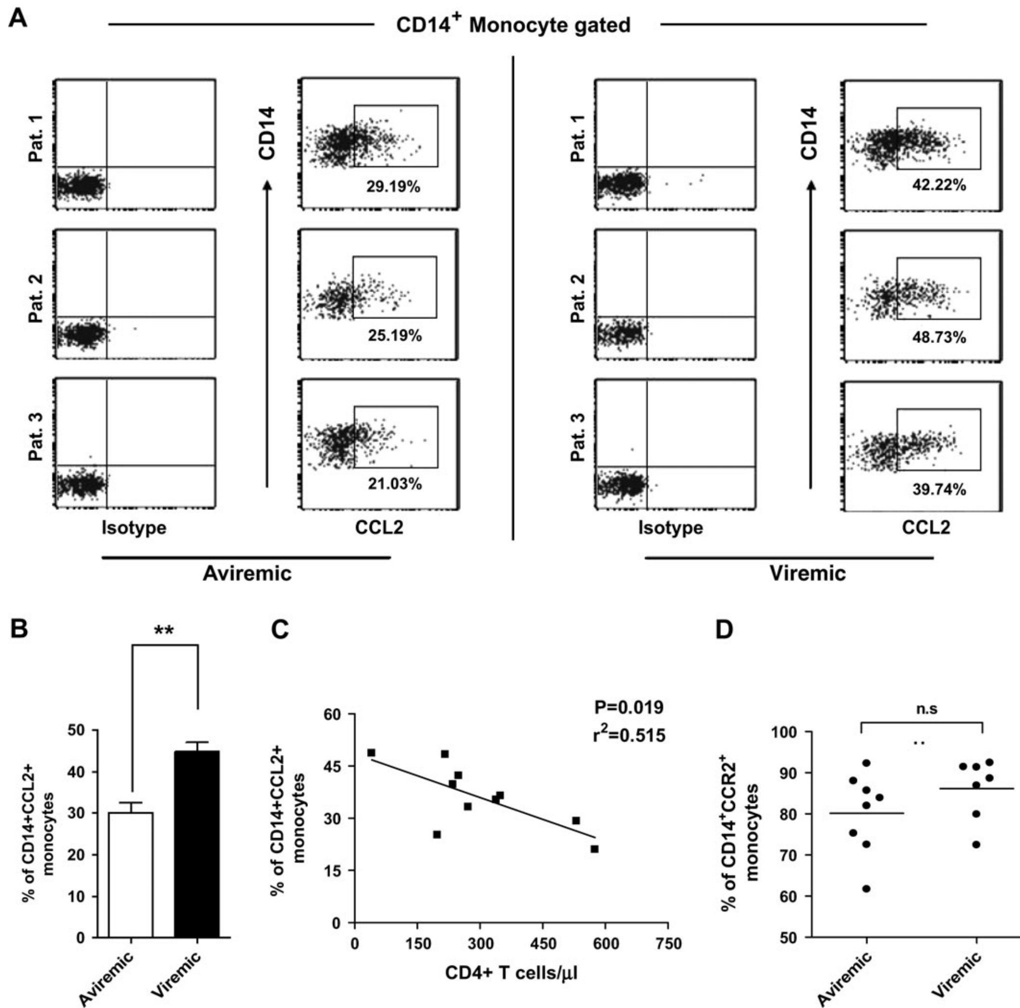

Fig. 4. Phenotypic analysis of HIV-1 patient-derived PBMC after *in vitro* culture. (A) Two-color staining was performed on frozen PBMC samples after 24 h of stimulation with PMA. Dot plots represent percentage of CD14+CCL2+ monocytes after gating on monocytes based on forward and side scatter gating. The left panel shows the isotype control in both aviremic and viremic plots and data shown here are representative of three patients from each group. (B) A higher percentage of CD14+ monocytes producing CCL2 is found in viremic (CD4+ T cells 191.8 ± 48.6 and viral load 24 482 ± 16 921, $n = 4$) as compared with aviremic (CD4+ T cells 377.3 ± 60.0 and viral load <50, $n = 6$) patients. (C) Association between CD4+ T cells and the percentage of CCL2-expressing CD14+ monocytes in PBMC of infected patients. (D) CCR2 expression on freshly isolated PBMC of aviremic (80.16 ± 3.48, $n = 8$) and viremic patients (86.15 ± 2.79, $n = 7$). CD14+CCR2+ monocytes were acquired after gating monocytes population. Each dot presents one individual. Data represented as mean ± SEM. $^{**}P < 0.005$ and n.s. stands for non-significant.

infection of PBMC obtained from four healthy volunteers. A very recent study using a mouse model of HIV-1 infection demonstrated CCL2 gene induction in brain tissues of infected animals (35). These data suggest that HIV-1 viremia alters the transcriptional program of the host cytokine/chemokine network, and this effect is most prominent with respect to CCL2 expression.

Transcriptional regulation could be translated into production of CCL2 because significantly elevated serum CCL2 concentrations were observed in HIV-1 viremic as compared with aviremic or healthy individuals. Furthermore, we could demonstrate a positive correlation between viremia and serum CCL2. Interestingly, an inverse correlation of CD4+ T cells was associated with more CCL2-expressing CD14+ monocytes. Since CCL2 is a strong lymphocyte chemoattractant, its higher production by host cells upon HIV-1 infection may result in recruitment of more target cells to the site of infection, resulting in higher HIV-1 replication and rapid spreading of the virus (Fig. 5). We can assume that the concentration of CCL2 at the site of infection is much higher than in the periphery; hence, a strong recruitment of CCR2-expressing target cells can be expected. CCR2, the receptor for CCL2, is expressed on the majority of monocytes and to a lesser extent on CD4+ T cells. However, we could not observe any significant effect of viremia on CCR2 expression on these cells, suggesting CCL2 concentration-dependent but surface CCR2-independent recruitment of target cells to the site of infection. Higher CCL2 expression might be at least in part mediated by TNF-α since it is a well-known inducer of CCL2, and higher TNF-α levels are associated with viremia as reported in many studies. Moreover, prior exposure of monocytes or monocyte-derived macrophages to β-chemokines has already been shown to enhance viral replication (36). Treatment with the HIV-1 protease inhibitor indinavir reduces the plasma CCL2 level in

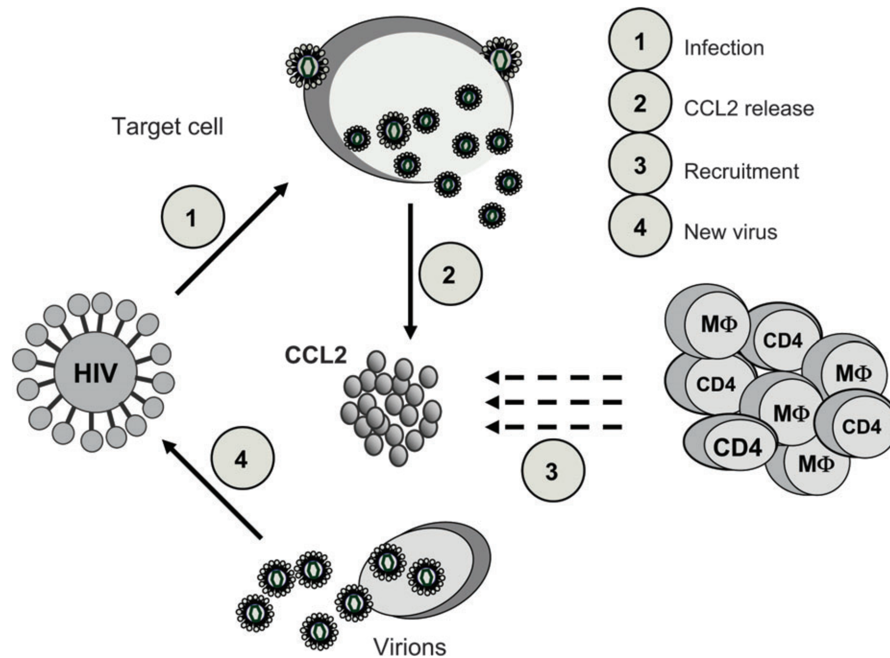

Fig. 5. Schematic view of proposed model for role CCL2 in HIV-1 replication *in vivo*. Feed back loop mechanism of CCL2-mediated HIV-1 replication. Stepwise events and involvement different cell types and cellular factors in depicting the *in vivo* role of CCL2 in virus replication are shown. Macrophages (MΦ) and CD4+ T cells (CD4) are shown as major target cells. Infection of HIV-1 induces CCL2 expression that recruits CCR2-bearing target cell, mostly monocytes and CD4 T cells, to the site of infection. These freshly recruited cells make a favorable niche for virus infection and replication resulting into increased viral load.

severely immunodeficient HIV-infected individuals (37). This effect is most likely explained by reduced viral replication due to effective treatment with indinavir. A higher level of CCL2 has been previously reported in HIV-1 patients plasma (38); however, there was no discrimination between viremic and aviremic patients. Here, we report that CCL2 is regulated not only at the protein but also at the mRNA level in HIV-1-infected viremic individuals. The potential role of CCL2 in HIV-1 replication *in vitro* can be explained in PHA blast model of HIV-1_{IIIB} infection. We observed >2-fold increase in the viral titer after 5 days of post-infection (data not shown), which is in line with previous reports (23, 39). Although we are not providing direct evidence for CCL2 in virus replication, the studies presented here strongly suggest that CCL2 is an integral host factor in HIV-1 pathogenesis, supporting the hypothesis that CCL2 presumably could act as a potential enhancer of HIV-1 replication.

In summary, our results from gene expression profiling and increased production of CCL2 in HIV-1 viremic individuals suggest that active HIV-1 replication, as manifested in viremic patients, at least in part is mediated by the inflammatory chemokine CCL2. Our data show a clear correlation between the viremia and host CCL2 gene response. Unraveling the mechanism of increased HIV-1 replication and potential involvement of CCL2 could provide more insight to understand the virus–host relationship, and inhibition of increased CCL2 production may lead to new therapeutic interventions in HIV-1 infection.

Supplementary data

Supplementary figures are available at *International Immunology* Online.

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Abbreviations

APC	allophycoerythrin
CCL2	C-C chemokine ligand-2
CCR1	C-C chemokine receptor-1
CXCL10	CXC chemokine ligand-10
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GCH1	GTP-cyclohydrolase-1
HAART	highly active anti-retroviral therapy
IP	inducible protein
MCP	macrophage chemoattractant protein
MIP	macrophage inflammatory protein
PMA	phorbol myristate acetate
RANTES	regulated upon activation, normal T cell expressed and secreted
TNF	tumor necrosis factor

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