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Stimulation of Cell Surface CCR5 and CD40 Molecules by Their Ligands or by HSP70 Up-Regulates APOBEC3G Expression in CD4+ T Cells and Dendritic Cells

Jeffrey Pido-Lopez,* Trevor Whitall,* Yufei Wang,* Lesley A. Bergmeier,‡ Kaboutar Babaahmady,* Mahavir Singh,§ and Thomas Lehner2*

Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like-3G (A3G) is an intracellular innate antiviral factor that deaminates retroviral cytidine to uridine. In an attempt to harness the anti-HIV effect of A3G, we searched for an agent that would up-regulate A3G and identify the receptors involved. Stimulation of cell surface CCR5 with CCL3 and CD40 with CD40L or both molecules with microbial 70-kDa heat shock protein (HSP)70 up-regulated A3G mRNA and protein expression in human CD4+ T cells and monocye-derived dendritic cells (DC), demonstrated by real-time PCR and Western blots, respectively. The specificity of CCR5 and CD40 stimulation was established by inhibition with TAK 779 and mAb to CD40, as well as using human embryonic kidney 293 cells transfected with CCR5 and CD40, respectively. A dose-dependent increase of A3G in CCL3- or HSP70-stimulated CD4+ T cells was associated with inhibition in HIV-1 infectivity. To differentiate between the inhibitory effect of HSP70-induced CCR5 binding and that of A3G, GFP-labeled pseudovirions were used to infect human embryonic kidney 293 cells, which showed inhibition of pseudovirus uptake, consistent with A3G being responsible for the inhibitory effect. Ligation of cell surface CCR5 receptors by CCL3 or CD40 by CD40L activated the ERK1/2 and p38 MAPK signaling pathways that induced A3G mRNA expression and production of the A3G protein. These in vitro results were corroborated by in vivo studies in rhesus macaques in which A3G was significantly up-regulated following immunization with SIVgp120 and p27 linked to HSP70. This novel preventive approach may in addition to adaptive immunity use the intracellular innate antiviral effect of A3G.


The difficulties encountered with the development of a preventive or therapeutic vaccine against HIV-1 infection have focused attention to the innate arm of immunity (1–3). The innate immune response is rapid, does not rely on immunological memory, and may be involved in driving adaptive immunity. There are three components to innate immunity: cellular (dendritic cells (DC)), macrophages, NK, and γδ cells), which involve the TLR (4); extracellular factors (type I and II IFN, cellular antiviral factor, CC chemokines, and complement); and intracellular factors (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like-3G (A3G) (5), tripartite motif (6), and Murr1 (7)). Intracellular innate immunity against HIV-1 has acquired considerable importance since the discovery of A3G. This is an enzyme with a molecular mass of 46 kDa, which is packaged into retroviral virions and deaminates viral cytidine to uridine, rendering them nonfunctional and inhibiting viral replication. Recently, A3G has also been found to inhibit HIV by an additional mechanism, possibly at or before reverse-transcription (RT) stage of the viral RNA (8). This innate mechanism of resistance to retroviral infection is counteracted by the HIV-1 viral infectivity factor (Vif), which protects the virus by preventing incorporation of A3G into virions and by rapidly inducing its ubiquitination and proteasomal degradation (9–12).

A3G is found in human T lymphocytes, monocytes, and macrophages, and lethally hypermutates viral DNA shortly after it is synthesized by RT (11–15). A3G was also demonstrated in testes, ovaries, and a number of tumor cell lines (14). There is limited information concerning the factors that control expression of A3G, but its level of mRNA does not change with HIV-1 infection (11, 16). A3G inhibits both the R5 and X4 strains of HIV-1 (5). Phorbol esters up-regulate A3G mRNA in a T cell line (H9), and this is mediated by the protein kinase C/mitogen-activated protein/ERK signaling cascade (13). PHA also up-regulates A3G (11), whereas IFN-α and IFN-γ stimulate an increase in A3G in macrophages (13, 14), leading to inhibition of HIV-1 replication in these cells.

To prevent HIV-1 infection, we attempted to identify cell surface molecules that might be involved in stimulating A3G expression. We explored recent findings that 70-kDa heat shock protein (HSP)70 significantly inhibits HIV-1 infectivity of human CD4+ T cells (K. Babaahmady, M. Singh, and T. Lehner, manuscript in preparation), and that it elicits chemokine and cytokine functions by engaging CCR5 and CD40 molecules (17–19). Indeed, we have found that in vitro stimulation of the cell surface CCR5 and CD40 molecules up-regulates A3G in CD4+ T cells and DC. Immunization of rhesus macaques with HSP70 linked to SIV Ags also up-regulates A3G in PBMC, suggesting the application of HSP70.
or other CCR5 and CD40 ligands to preventative and therapeutic vaccination against HIV-1 infection.

Materials and Methods

Preparation of microbial HSP70

Recombinant Mycobacterium tuberculosis HSP70 was prepared from the Escherichia coli pop strain (20). HSP70 was purified by ion-exchange chromatography using Q-Sepharose, followed by ATP affinity chromatography. The HSP70 preparation was further treated with polyimix B-coated beads (Sigma-Aldrich) to remove LPS. The LPS content of the HSP preparations was determined by the Limulus amoebocyte lysate assay (Sigma-Aldrich), and showed <0.006 U/μg HSP70 or 5 pg/μl HSP70 preparation. A sample of LPS-free HSP70 was further excluded by calcium mobilization elicited by HSP70, but not LPS and protein kinase T treatment, which inhibited TNF-α production stimulated by HSP70, but not by LPS (data not presented; see Refs. 17 and 18). Furthermore, dose-dependent inhibition by pertussis toxin producing TNF-α and IL-12 was found with HSP70, but not with LPS. In many assays, LPS was used as a control, so as to exclude further the possibility that any LPS contaminant might have been responsible for the results (21, 22).

LPS, CD40L, CCL-3 (MIP-1α), CXCL-12, and TAK 779

LPS derived from E. coli strain 011B4 was purchased from Sigma-Aldrich. Soluble CD40L trimer was donated by F. Villinger (Department of Immunology, Emory University, Atlanta, GA). CCL3 (MIP-1α) and CXCL12 were purchased from R&D Systems. TAK 779 was obtained from the Medical Research Council AIDS Research Reagents Programme.

Preparation of human CD4+ and CD8+ T cells, monocytes, and DC

Volunteers were recruited from the staff and postgraduate students at Guy’s Hospital. Permission was obtained for this investigation from the ethics committees of Guy’s and St. Thomas’ Hospital Medical School. Approximately 50 ml of venous blood was taken, and PBMC was separated by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Nycomed). CD4+ and CD8+ T cells were enriched by depletion of CD14+ monocytes (Miltenyi Biotec), through a combination of Abs to CD11b, CD16, CD19, CD36, and CD56, with either CD4 or CD8 (CD8-T cell isolation kit, or CD4-T cell isolation kit; Miltenyi Biotec). Eluted cells that were not bound to the columns were enriched CD4-positive or CD8-positive T cells, and contained <5% of CD8+ or CD4-positive cells, respectively, and <1% monocytes. Human primary monocytes were isolated from PBMC prepared from healthy donors. CD14+ monocytes were enriched by depletion of CD14+ cells using the Monocyte Isolation Kit (MACS, Miltenyi Biotec). The purity of isolated monocytes was consistently >90% when analyzed by flow cytometry with Ab to CD14. Human DC were generated by culturing monocytes in RPMI 1640, 10% FCS, GM-CSF (400 U/ml), and IL-4 (100 U/ml) for 5 days. These monocyte-derived DC were considered to be immature DC, defined by surface expression of DC markers CD83, CD80, CD86, and CD40, and were CD14+. To generate mature DC, immature DC were further stimulated with 20 μg/ml HSP70, and for some experiments with 10 ng/ml LPS or 10 ng/ml CD40L for 48 h.

Cell lines and stimulation

Human embryonic kidney (HEK) cell line (HEK 293) and stably transfected HEK 293 cells with CCR5 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The cells showed 85% (mean fluorescence intensity (MFI) 173) CCR5 and were enriched by depletion of CD14+ monocytes (Miltenyi Biotec), through a combination of Abs to CD11b, CD16, CD19, CD36, and CD56, with either CD4 or CD8 (CD8-T cell isolation kit, or CD4-T cell isolation kit; Miltenyi Biotec). Eluted cells that were not bound to the columns were enriched CD4-positive or CD8-positive T cells, and contained <5% of CD8+ or CD4-positive cells, respectively, and <1% monocytes. Human primary monocytes were isolated from PBMC prepared from healthy donors. CD14+ monocytes were enriched by depletion of CD14+ cells using the Monocyte Isolation Kit (MACS, Miltenyi Biotec). The purity of isolated monocytes was consistently >90% when analyzed by flow cytometry with Ab to CD14. Human DC were generated by culturing monocytes in RPMI 1640, 10% FCS, GM-CSF (400 U/ml), and IL-4 (100 U/ml) for 5 days. These monocyte-derived DC were considered to be immature DC, defined by surface expression of DC markers CD83, CD80, CD86, and CD40, and were CD14+. To generate mature DC, immature DC were further stimulated with 20 μg/ml HSP70, and for some experiments with 10 ng/ml LPS or 10 ng/ml CD40L for 48 h.

Isolation of RNA

RNA was isolated from ~2 × 106 cells using a Total RNA Isolation Kit (Promega), and cDNA was generated from RNA by RT-PCR using the Reverse Transcription System (Promega), according to the manufacturer’s instructions.

Transfection

Full-length human CD40 cDNA in the pCDM8 plasmid vector (Invitrogen Life Technologies) was provided by B. Seed (Department of Genetics, Harvard University, Boston, MA). The HEK 293 T cells were cultured in 25-cm2 flasks until 30–50% confluence and transfected using Lipofectamine Plus (Invitrogen Life Technologies). Transfection was performed, according to the manufacturer’s protocol, and CD40 was detected by flow cytometry using FITC-conjugated CD40 mAb.

Receptor inhibition

CCR5-transfected HEK 293 cells were treated with 0, 10, 50, 100, or 500 nM TAK 779. Stably CCR5 overexpressing HEK cells were stimulated with 20 μg/ml HSP70 for 18 h. CD40-transfected HEK 293 T cells were treated with 0, 15 μg/ml anti-CD40 Ab for 1 h, and then stimulated with 20 μg/ml HSP70 for 18 h.

PCR of A3G

Aliquots of 100 ng of the cDNA were added to a PCR mix containing Taq polymerase, reaction buffer, 3 mM MgCl2, 0.2 mM dNTP, and 12 μM each A3G primer (5′-TACAGGGTTACCTGCTACCCCT-3′ and 5′-AAG TAATGCGACTCTAATTTTTA-3′). PCR conditions were 95°C for 5 min, then 90°C for 1 min, 57°C for 1 min, and 72°C for 2 min for 32 cycles, and then 72°C for 10 min. PCR products (500 bp long) were run on a 1% agarose gel containing 0.001% ethidium bromide. Samples were normalized using GAPDH PCR amplification as an endogenous control. Inhibition of HSP70-stimulated A3G was by treating the cells with mAb to CD40 (1.5 μg/ml) and control with the IgG2a isotype (1.5 μg/ml).

Real-time PCR of A3G mRNA

A3G mRNA expression was determined by real-time PCR using the ABI Prism 5700 (Applied Biosystems). Approximately 100 ng of cDNA was added in duplicate to a PCR mix containing TaqMan Universal Mastermix (Applied Biosystems) and commercially available A3G primers and fluorescence labeled probes (Assays on Demand; Applied Biosystems). The cDNA was amplified on the ABI Prism 5700, according to the manufacturer’s instructions. Real-time PCR amplification within samples was normalized using GAPDH amplification (23). GAPDH primers and fluorescent labeled probes were obtained from Assays on Demand (Applied Biosystems). The sensitivity of the A3G and GAPDH PCR was initially tested and validated by amplification of the target product from serially diluted cDNAs. A difference in the cycle threshold (ct) value by cycles at every log10 dilution of the cDNA concentration and similar and parallel trends of the curves for GAPDH and A3G reaction graphs (slope ≤0.1) were taken as confirmation of the validity of the GAPDH and A3G PCR (data not shown). Data analysis was performed following the 2-ΔΔct comparative method outlined in the ABI Prism user bulletin to obtain the fold difference in mRNA expression between two different samples, which is stimulated relative to unstimulated cells.

Western blots of A3G protein

To detect A3G protein, 2 × 106 cells were treated for 24 h with HSP70 (25 μg/ml), CD40L (1 μg/ml), or PHA (10 μg/ml), and then lysed in HBBS, with 10 mM HEPES (pH 7.4) with 1% Nonidet P-40. Lysates were cleared by centrifugation, and an equal volume of SDS sample buffer was added before SDS-PAGE under reducing conditions. For the experiments with macaque cells and comparison of different cells, A3G was immunoprecipitated using polyclonal rabbit anti-human A3G (Immunodiagnostics) and protein G-Sepharose (Amersham Pharmacia). Bound A3G was released by addition of SDS sample buffer for SDS-PAGE, as above. After transfer of proteins to a polyvinylidene difluoride membrane, Western blotting was conducted with mouse mAb to A3G (Immunodiagnostics) or β-actin (Sigma-Aldrich), using biotinylated anti-mouse Ab, streptavidin-peroxidase, and ECL-plus reagents (Amersham).

Signaling

To study the signaling pathway, DC were incubated with 20 μM p38 MAPK inhibitor SB 203580 or 10 μM ERK1/2 inhibitor PD 098059 (both Sigma-Aldrich) for 30 min and stimulated with HSP70 (20 μg/ml) or PHA
A3G expression was determined. β-Actin was used as a loading control for A3G, and the cells were checked for direct toxic effect of the inhibitors by trypan blue, which showed no impaired viability of the cells.

The effect of HSP70 on HIV-1 infectivity

The CCR5 strain of HIV-1 (BaL) isolate was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. PBMCs were stimulated with PHA (Sigma-Aldrich) and IL-12 (Schiaiparelli Biosystems), using standard protocol to grow the virus. RT activity in the culture supernatants was monitored using Quan-T RT kit (Amersham Biosciences), and the virus was harvested at a peak time point. The-HIV-1BaL stock titers were determined by RT, and the 50% tissue culture infective dose of the virus was then determined.

To test HIV-1 infectivity, we separated CD4-positive T cells from PBMC using the CD4+ T cell separating kit (Miltenyi Biotec). CD4-positive cells were activated with 10 µg/ml PHA in RPMI 1640 medium supplemented with penicillin, streptomycin, 10% FCS, and 20 IU of IL-2 (Schiaiparelli Biosystems) for 3 days, and then washed with medium and counted. Cells were infected with primary HIV-1 BaL (CCR5-binding strain), using 100 µl (20,000 cpm) of the virus to infect 10⁶ cells. They were incubated for 3 h and washed with medium and counted.

In vivo studies of A3G in immunized macaques with HSP70

PBMC were separated from the blood of five groups of rhesus macaques in which blood was taken 2–4 wk after the last of three immunizations. Group 1 unimmunized animals (n = 3) were compared with group 2 (n = 3), immunized with SIVgp120 (200 µg) and gag p27 (200 µg) covalently linked to HSP70 (200 µg). Group 3 macaques (n = 3) were immunized with SIVgp120 and gag p27 (100 µg each), covalently linked to HSP70 (200 µg); in addition to comparing pre- and postimmunization PBMC, they were also compared with group 4 macaques (n = 3) immunized with alum (200 µg Alugel; Uniscience) adsorbed with HIVgp140 (100 µg) and SIV gag p27 (200 µg). Increase in A3G was assayed by real-time PCR in PBMC from naive macaques, HSP70/SIV Ags, alum-HIV/SIV Ags, and HSP70-immunized animals without Ags (group 5, n = 2). The animals were housed and handled in accordance with the U.K. Home Office and European guidelines.
FIGURE 2. Evidence that stimulation of CCR5 with CCL3 (10 ng/ml) or HSP70 (20 μg/ml) up-regulates A3G expression. A, Dose-dependent effect of CCL3 compared with CXCL12 (10 ng/ml) on expression of A3G (***, p < 0.0001); B, inhibition of HIV-1 infectivity in primary CD4+ T cells; C, mRNA in DC stimulated with CCL3 compared with CXCL12 and HSP70 (n = 4; *, p = 0.04; **, p = 0.002); D, inhibition of CCR5-mediated production of A3G protein by TAK 779 in DC stimulated with CCL3 or HSP70 compared with the CD40L control and β-actin; E, mRNA in CCR5+ compared with CCR5- HEK 293 cells stimulated with CCL3 (n = 4; p = 0.015) or HSP70 (n = 9; **, p = 0.006); and F, inhibition by TAK 779 of A3G mRNA in CCR5+ compared with CD40+ HEK 293 cells stimulated with HSP70 (*, p = 0.057; **, p = 0.0001).

Preparation of the HIV-GFP virus

To prepare GFP-labeled HIV-1, HEK 293 T cells were transfected with plasmids p8.91 (encoding for HIV-1 gag, pol, tat, and rev), pCSGW (encoding for GFP), and pVSV-G (encoding for vesicular stomatitis virus (VSV) viral envelope) provided by M. Malim and K. Bishop (Kings College, London, U.K.) (24). The VSV viral envelope avoided the requirement of CD4 and HIV coreceptors for cell entry by virions. The transfection was conducted by the lipofectin (Invitrogen Life Technologies) method, according to the manufacturer’s instructions. Virions were harvested 3–4 days posttransfection and normalized by p24 ELISA (Gentaur).

To infect HEK 293 cells expressing CCR5, −5 × 10^5 cells/well, in 0.5 ml of DMEM with 10% FCS, were plated into 12-well plates and treated with either HSP70 (20 μg/ml) or LPS (10 ng/ml) and incubated at 37°C and 5% CO2 for 18 h. The cells were infected with 1 ml of GFP-virus extract, containing 30–50 pg/ml p24, and incubated for 3 days. Cells were then harvested and assessed for viral infection by GFP expression using FACS analysis.

Real-time PCR of rhesus macaque A3G mRNA

PBMC (3 × 10^6, 500 μl/well) were distributed into 24-well plates and incubated in RPMI 1640 (supplemented with 10% FCS, 2 μM glutamine, and 100 μg of penicillin and streptomycin) for 18 h with 10 μg/ml HSP70 or 10 μg/ml PHA. The cells were then harvested and washed with PBS. The RNA was extracted using a Total RNA kit, and cDNA was prepared using the RT system (Promega), as described above.

Quantification of A3G mRNA was conducted by real-time PCR (ABI Prism 5700) using the PlatinumSYBR green qPCR SuperMix-UDG with ROX (Invitrogen Life Technologies). The primers used for rhesus macaques A3G were as follows: 5'-CTG TCC ACT GAC CAC ACG TT-3' (right); 5'-ACA TGC CAC GAA GAT CA-3' (left). The amplification conditions were 2 min at 50°C, 2 min at 95°C, 40 cycles of 15 s at 95°C, and 30 s at 60°C. The results were standardized within samples using rhesus macaques GAPDH mRNA (primers: 5'-GGA CTC CTC CAG AAC ATC ATC CCT-3' (right); 5'-CCT TGA GGG GCC CCT CGG ACG CCT-3' (left)). Melting curve analysis was performed in every assay, and the products were also analyzed on agarose gel to confirm the specificity of amplified products. The data analysis was described above.

Statistics

The results are expressed as mean (±SEM) and were analyzed with the appropriate Student t test.

Results

Stimulation of mRNA and protein of A3G by HSP70 in primary CD4+ T cells and the effect on HIV-1 infectivity

The rationale for exploring HSP70 as an agent that will stimulate A3G production was based on the inhibitory effect of HSP70 on HIV-1 replication (K. Babaahmady, M. Singh, and T. Lehner, manuscript in preparation). Dose-dependent increase in A3G mRNA expression was demonstrated by real-time PCR in primary CD4+ T cells stimulated with HSP70 (Fig. 1A), and these cells showed corresponding inhibition of HIV-1 infectivity (Fig. 1B), using an R5 strain of HIV-1 (BaL). Increased A3G mRNA (Fig. 1C) and protein (Fig. 1D) expression were also found after HSP70 stimulation of CD4+ T cells and monocyte-derived immature DC (Fig. 1, E and F), respectively. The GAPDH control showed little or no change (Fig. 1, C and E). Thus, stimulation by HSP70 induced a dose-dependent increase in A3G mRNA and protein expression that was associated with inhibition of HIV-1 infection in CD4+ T cells and monocyte-derived DC.

Inhibition of GFP-labeled HIV-1 virions

To differentiate between HSP70 binding CCR5 directly or via CC chemokines and A3G, we used single-round infection assay by GFP-labeled HIV-1 pseudovirions to infect CCR5+ HEK 293
cells. Before pseudovirion exposure, the CCR5+ HEK 293 cells were treated overnight with either 20 μg/ml mHSP70 or 10 ng/ml LPS. FACs analysis of cells 3 days postexposure to the GFP+ virions revealed that the percentage of GFP+ cells was 5- to 6-fold greater for the untreated (44.2%; MFI 176.2) or LPS-treated cells than for the HSP70-treated cells (8.6%; MFI 20.3; p = 0.006) (Fig. 2E). The specificity of HSP70-binding CCR5 was confirmed by treatment of the CCR5-transfected HEK 293 cells with TAK 779, which induced greater A3G expression (4.0 ± 0-fold) than stimulating untransfected cells (1.3 ± 0.3-fold; p = 0.015), whereas stimulation with HSP70 showed greater A3G expression in CCR5-transfected cells (13.3 ± 3.0-fold) than untransfected cells (1.9 ± 1.1-fold; p = 0.006) (Fig. 2E). The specificity of HSP70-binding CCR5 was confirmed by treatment of the CCR5-transfected HEK 293 cells with TAK 779, which showed a significant dose-dependent inhibition of HSP70-induced A3G expression, unlike the CD40-transfected cells (Fig. 2F).

**Up-regulation of A3G expression mediated by stimulation of the CCR5 molecules**

Having prior knowledge that the CCR5 and CD40 molecules mediate HSP70 stimulation in CD4+ T cells and DC (17–19), we first examined the possibility that ligation of CCR5 will up-regulate A3G. Stimulation of primary human CD4+ T cells (Fig. 2A) or immature DC (data not presented) with CCL3 showed a dose-dependent increase in A3G mRNA expression, compared with CXCL12, which does not bind CCR5 and showed no change. A corresponding dose-dependent inhibition of HIV-1 infectivity was found when the primary CD4+ T cells were treated with CCL3, but not CXCL12 (Fig. 2B). The effect of CCL3 (10 ng/ml) was then compared with HSP70 (20 μg/ml) in immature DC, which showed an increase in A3G expression (4.8 ± 0.8- and 5.6 ± 0.9-fold, respectively), whereas CXCL12 (10 ng/ml) showed negligible increases in A3G (1.7 ± 0.25; p = 0.002 and 0.04, respectively; Fig. 2C). A3G protein was also up-regulated following treatment with the above concentration of HSP70 and to a lesser extent with CCL3 and CD40L, but only CCL3- and HSP70-stimulated increases in A3G generation were inhibited by TAK 779, a specific CCR5 inhibitor, as CD40L-stimulated increase in A3G is mediated by CD40 and not CCR5 molecules (Fig. 2D). The β-actin control remained largely unchanged. CCR5-mediated stimulation of A3G expression was further supported by stimulating CCR5-transfected HEK 293 cells with CCL3, which induced greater A3G expression (4.0 ± 0-fold) than stimulating untransfected cells (1.3 ± 0.3-fold; p = 0.015), whereas stimulation with HSP70 showed greater A3G expression in CCR5-transfected cells (13.3 ± 3.0-fold) than untransfected cells (1.9 ± 1.1-fold; p = 0.006) (Fig. 2E). The specificity of HSP70-binding CCR5 was confirmed by treatment of the CCR5-transfected HEK 293 cells with TAK 779, which showed a significant dose-dependent inhibition of HSP70-induced A3G expression, unlike the CD40-transfected cells (Fig. 2F).

**Up-regulation of A3G expression mediated by stimulation of the CD40 molecules**

We then studied CD40, which is engaged by the CD40L (CD154), and is an important molecule in the costimulatory pathway of cognate immunity (25). Indeed, stimulation of DC with CD40L resulted in a significant dose-dependent increase in A3G, which was not found when DC were stimulated with LPS (***, p = 0.02; Fig. 3A). A comparative study of stimulating DC with CD40L or HSP70 showed 5.4 ± 1.9-fold increase in A3G expression with CD40L, which was similar to that stimulated by HSP70 (6.8 ± 1.8-fold; Fig. 3B). This was supported by significant CD40L-stimulated increase in A3G mRNA expression in CD40-transfected (4.0 ± 0-fold), compared with untransfected HEK 293 T cells...
A significant increase in A3G expression was also found following HSP70 stimulation of CD40-transfected (8.0 ± 2.3-fold), compared with untransfected (1.0 ± 0.03; \( p = 0.022 \)) HEK 293 T cells (Fig. 3C). Furthermore, inhibition of HSP70-stimulated A3G mRNA expression and protein in monocyte-derived DC was demonstrated using mAb to CD40 by PCR (Fig. 3D). Examination of A3G protein by Western blots also showed increases in A3G production stimulated by CCL3, HSP70, or CD40L (Fig. 3E), and treatment with CD40 Abs inhibited CD40L- and HSP70- but not CCL3-stimulated up-regulation of A3G. The control β-actin showed little change, except for an increased concentration with CD40L and anti-CD40 treatment, which, however, was converse to the A3G expression (Fig. 3E). These results in monocyte-derived DC and CD40-transfected 293 cells suggest that activation of the CD40 cell surface molecules induces A3G expression.

**Signaling by the ERK1/2 and p38 phosphorylation pathways**

The signaling pathway of A3G stimulation mediated by CCR5 and CD40 was then explored. Treatment of HSP70- or PHA-stimulated DC with ERK1/2 (PD09859) or p38 (SB203580) inhibitor showed a decrease in A3G protein, compared with untreated DC (Fig. 3F). These results are consistent with ERK1/2 and p38 phosphorylation pathways being involved in the CCR5- and/or CD40-mediated activation of A3G expression.

**Comparative study of mRNA and protein expression of A3G in five subsets of human PBMC**

A comparative study of mRNA and protein expression of A3G was then pursued in CD4+ and CD8+ T cells, monocytes, and immature and mature DC. Resting CD8+ T cells expressed more A3G protein than CD4+ T cells (Fig. 4A). Stimulation with HSP70 for 28 h resulted in an increase in A3G, which was relatively greater in CD4+ than CD8+ T cells. A similar comparison of the monocyte and immature and mature DC series demonstrated greater protein levels of A3G in the unstimulated monocytes and DC than in the T cell subsets (Fig. 4A). HSP70 stimulation again resulted in increased expression of A3G in all three subsets of monocytes and DC. Real-time PCR was then used to assess quantitatively the effect of HSP70 stimulation on the expression of A3G mRNA. A3G was up-regulated with a mean (±SEM) fold increase of 1.7 (±0.21) in CD8+ T cells and 3.0 (±0.4) in CD4+ T cells (\( p = 0.04 \); Fig. 4B). Very significant increase in HSP70-stimulated A3G mRNA expression was found in immature (6.8 ± 1.3) and mature DC (11.2 ± 2.5), compared with monocytes (1.7 ± 0.4) (\( p = 0.001 \); Fig. 4B). Clearly, a direct association between A3G mRNA and protein levels was not observed for all of the cell subsets examined. Stimulation of the CD4+ or CD8+ T cells and immature DC with HSP70 was then compared with that of LPS (Fig. 4C). The results suggest that A3G in CD4+ T cells is significantly up-regulated with HSP70 (3.0 ± 0.45), compared with LPS (1.25 ± 0.25; \( p = 0.014 \)), and this was also found to a lesser extent with CD8+ T cells (\( p = 0.013 \); Fig. 4C). Immature DC also showed significantly greater increase in A3G when stimulated with HSP70 (6.9 ± 1.84) than with LPS (2.4 ± 0.43; \( p = 0.04 \)) (Fig. 4C). Thus, HSP70 is significantly more potent than LPS in up-regulating A3G in all three cell subsets. Furthermore, when LPS or CD40L was used for maturation of DC and these were then stimulated with HSP70, comparable A3G expression was found to that resulting from inducing DC maturation with HSP70 (data not presented).

**In vivo up-regulation of A3G following immunization with HSP70 in rhesus macaques**

The in vitro studies were followed by a retrospective in vivo investigation of five groups of rhesus macaques. PBMC from naive animals (group 1) was compared with those from HSP70 linked to SIVgp120- and p27-immunized (×3) macaques (group 2). 2–4 wk after the last immunization to determine whether A3G can be up-regulated in vivo. A3G mRNA and the protein showed increases in HSP70-immunized compared with the unimmunized macaques (Fig. 5, A and B), suggesting that HSP70 immunization induces an increase in A3G expression. This was confirmed by real-time PCR analysis, which showed a mean increase of 3.2 ± 0.12-fold in A3G expression in the cells from immunized, as compared with those from naive (1.0 ± 0.0) (\( p = 0.07 \)) animals (Fig. 5C). Further studies of A3G were then pursued before and 2–4 wk after immunization in a third group of macaques immunized with HSP70 linked to SIVgp120 and p27, and compared with a fourth group of macaques given alum-adsorbed SIVgp120 and p27. Whereas PBMC of the HSP70 vaccine-immunized macaques showed significant fold increase in A3G expression of 5.1 ± 0.48 after immunization relative to the preimmunization level (\( p = 0.013 \), the
alum-immunized macaques showed little or no change (1.2 ± 0.1; Fig. 5C). This was corroborated by the conventional PCR (Fig. 5A). A comparison between SIV Ags linked to HSP70 and those adsorbed to alum showed a significant increase in A3G expression with HSP70 (p = 0.032). Additional in vitro stimulation with HSP70 of PBMC from naive macaques showed an increase in A3G (3.8 ± 1.0) (Fig. 5D), but PBMC from the HSP70-immunized macaques showed only a slight further increase in A3G (4.1 ± 1.3) over the level without in vitro treatment with HSP70 (3.2 ± 0.12). Similarly, in vitro stimulation with HSP70 of PBMC from the third group of macaques showed an increase in A3G that was greater in the pre- than in postimmunized macaques (Fig. 5, D compared with C), unlike the alum-immunized animals that showed only a slight increase in A3G with HSP70. Thus, the increase in A3G can be attributed to immunization with HSP70, as immunization with HIVgp120 and SIV p27 adsorbed to alum failed to up-regulate A3G (1.2 ± 0.09) (Fig. 5C). This is consistent with immunization of two macaques with HSP70 alone, which also up-regulated A3G (mean 3.8; Fig. 5E), and showed no further increase in A3G following restimulation with HSP70 in vitro, unlike in the naive animals (Fig. 5F).

**Discussion**

It has been established that the HIV-1-inhibitory activity of A3G is neutralized by Vif (9–12). This interaction has raised therapeutic potentials, by either inhibiting Vif activity or boosting A3G levels above the neutralizing capacity of Vif to inhibit virus replication (5, 26). The present studies have been confined to exploring the potential of up-regulating A3G expression first in vitro and then in vivo. The overall results suggest that up-regulation of A3G expression can be mediated by the CCR5 and CD40 molecules, which were stimulated by the chemokine CCL3 and CD40L (CD154), respectively, as well as by HSP70. This was supported by the finding that A3G mRNA expression is up-regulated following stimulation of either CCR5- or CD40-transfected, but not untransfected cell lines, and by the corresponding ligands and HSP70. The primary human cells that showed significant increase in A3G expression when stimulated with CCL3 or HSP70 were CD4+ T cells (3.0 ± 0.4) and immature (6.8 ± 1.3) and mature DC (11.2 ± 2.5), which serve as the major cellular targets of HIV-1. Macrophages were not studied in this work, but show an increase in A3G expression upon stimulation with IFN-α (14).

The specificity of CCR5 stimulation was confirmed by the lack of response of CCR5− HEK 293 cells or by stimulation with CXCL12, as well as the dose-dependent inhibition of A3G by TAK 779. These findings suggest that CCL3 may not only block and down-regulate CCR5, thereby inhibiting pre-entry of HIV-1 (27, 28), but also stimulate A3G expression, which inhibits postentry replication of HIV-1. Thus, HIV-1 inhibition with CCL3 or HSP70 cannot be ascribed solely to up-regulation of A3G expression. The GFP-labeled virions infecting CCR5+ HEK 293 cells are independent of CD4 and CCR5 expression due to the presence of a VSV envelope, so inhibition of the viral infection is most likely to be due to the intracellular A3G generated by stimulation with HSP70. Furthermore, the pseudo virus features only a single round of infection, which suggests that the A3G-inhibitory effect occurs during the early stages of infection, as observed recently with the low molecular mass (LMM) form of A3G (8). Interestingly, there is evidence that LMM A3G may be resistant to the actions of Vif,
thus making it more desirable as an anti-HIV agent than its non-Vif-resistant high molecular mass A3G counterpart (8). Whether HSP70 up-regulates only the LMM or both forms of A3G requires further investigation. The A3G-inhibiting mechanism involves deaminating deoxycytidine to deoxyuridine of the minus viral ssDNA during RT, and this enzymatic editing of HIV-1 reverse transcripts induces degradation of deaminated minus strand DNA, leading to inhibition of HIV-1 replication (9, 10, 29, 30).

Unlike CCR5, CD40 molecules are expressed on immature and mature DC, Langerhans cells, and macrophages, but not on CD4+ T cells. The specificity of the CD40-mediated response has been demonstrated by A3G mRNA up-regulation in CD40+T, but not CD40−HEK 293 T cells and inhibition of A3G mRNA and protein expression by mAb to CD40. The signaling pathway following stimulation of cell surface CD40 by CD40L or CCR5 by CCL3 was then studied, and this suggested that activation of the ERK1/2 and p38 MAPK signaling pathway induces A3G mRNA expression, which is translated to A3G production. Because the CD40L-CD40 costimulatory pathway between CD4+ T cells and APCs is essential in adaptive immunity between TCRs and peptide-HLA Ags (25), the finding that CD40 stimulation may increase expression of A3G is important. It has been well recognized that activation of CD4+ T cells enhances HIV-1 infectivity, raising concern that immunization might facilitate HIV-1 infection. The finding that CD40 activation by CD40L up-regulates A3G is arguably the first evidence that the enhanced infectivity of activated cells may be counteracted by concomitant increase in A3G expression. Indeed, CD40L is expressed by Ag-specific CD4+ T cells (31), which bind CD40 on DC and probably macrophages that may up-regulate A3G in these cells. Whether a reciprocal stimulation of A3G can be elicited in CD4+ T cells remains to be determined.

The mechanism for up-regulation of A3G can thus be activated by CCL3 stimulating CCR5, CD40L stimulating CD40, or HSP70 stimulating both molecules. An increased expression in CCR5 molecules does not take place; indeed, CC chemokines bind and down-regulate CCR5 on CD4+ T cells or CCR5-transfected Chinese hamster ovary cells (27, 28) and HSP70 down-regulates CCR5 on mature DC (18). However, CD40 is up-regulated in stimulating immature DC with HSP70, so it is possible that the greater cell surface expression of CD40 on mature DC might enhance activation of these cells. Nonetheless, a common MAPK signaling pathway for CCR5 and CD40 molecules involves activation of ERK1/2 and p38, which induce A3G mRNA expression and is translated to protein production. Persistence of A3G expression 2–4 wk after the last immunization with the HSP70-SIV vaccine raises at least two possibilities, as follows: 1) that HSP70 is retained by macrophages and when they undergo necrosis HSP70 is released and bystander macrophages or DC are stimulated to produce A3G (32); 2) that A3G production by CD4+ T cells or DC may retain an immune-like memory, despite it being considered to be an innate immune factor. Both these possibilities will be actively investigated.

The comparative investigation of A3G in the CD4+ and CD8+ T cells showed that whereas resting CD8+ T cells expressed more A3G protein than CD4+ T cells, stimulation of these cells with HSP70 resulted in significantly greater up-regulation of A3G mRNA and protein in CD4+ than CD8+ T cells. A somewhat similar relationship in A3G mRNA was revealed between monocytes and DC, in that HSP70 stimulation induced significantly greater increase in A3G mRNA expression in both immature and mature DC than monocytes. Further quantitative studies will need to be pursued to ascertain whether the final A3G protein levels have reached maximum expression in each of the five subsets of cells studied. The differential effect on HIV-1 infectivity between the CD4+ CCR5+ immature and CD4+ CCR5− mature DC is also of interest, as the maximal expression of A3G mRNA in mature DC is consistent with lack of HIV-1 infectivity (in contrast to transient carriage) of mature compared with immature DC. Furthermore, there are differences both in timing and magnitude of A3G up-regulation in CD4+ T cells and monocyte-derived DC, suggesting that A3G is more readily up-regulated in DC than CD4+ T cells. This finding might be significant in primary cervicovaginal and rectal mucosal transmission of HIV-1 by Langerhans cells and DC that migrate to the regional lymphoid tissue and infect CD4+ T cells (33–35).

The potential application of HSP70 as an A3G up-regulating agent in both preventive and therapeutic immunization was studied in vivo in rhesus macaques. Indeed, HSP70 alone or covalently linked to HIV or SIV Ags administered by the mucosal or systemic route significantly up-regulated A3G mRNA and protein in PBMC of macaques, whereas alum mixed with HIV and SIV Ags failed to affect A3G expression. Whether other adjuvants will affect the expression of A3G needs to be examined. The overall results suggest that HSP70 might be used as an HIV-designer adjuvant in preventive and therapeutic vaccination, as HSP70 may inhibit both pre-entry transmission of HIV-1 into CD4+ T cells or DC by generating CC chemokines (17, 18), and postentry replication of HIV-1 by up-regulating A3G. Whereas the conventional concept of innate immunity has been that it lacks memory, this may not apply to all innate immune responses, as demonstrated recently with NK cells in delayed hypersensitivity (36). It seems that the CC chemokines, CCL3, CCL4, and CCL5, are increased by repeated immunization with SIVgp120 and p27 in macaques (37), but the effect of repeated in vivo immunization on A3G expression will need to be studied. An important question, whether up-regulation of A3G following HSP70 stimulation can be maintained beyond the 4 wk shown in the present studies, will have to be determined, although preliminary indications are that this might be achieved. Thus, stimulation of the CCR5 molecule, which is the major coreceptor of R5 strains of HIV-1 (38, 39), or the CD40 (25) molecule, which is part of a major costimulatory pathway, up-regulates A3G and results in postentry inhibition of HIV-1 replication. This novel finding may be important in enhancing the innate intracellular anti-HIV-1 response and provides a complementary strategy in protective and therapeutic immunization against HIV-1.

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Disclosures
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References


