Increased expression with differential subcellular location of cytidine deaminase APOBEC3G in human CD4+ T-cell activation and dendritic cell maturation

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APOBEC3G (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G; A3G) is an innate defense protein showing activity against retroviruses and retrotransposons. Activated CD4+ T cells are highly permissive for HIV-1 replication, whereas resting CD4+ T cells are refractory. Dendritic cells (DCs), especially mature DCs, are also refractory. We investigated whether these differences could be related to a differential A3G expression and/or subcellular distribution. We found that A3G mRNA and protein expression is very low in resting CD4+ T cells and immature DCs, but increases strongly following T-cell activation and DC maturation. The Apo-7 anti-A3G monoclonal antibody (mAb), which was specifically developed, confirmed these differences at the protein level and disclosed that A3G is mainly cytoplasmic in resting CD4+ T cells and immature DCs. Nevertheless, A3G translocates to the nucleus in activated-proliferating CD4+ T cells, yet remaining cytoplasmic in matured DCs, a finding confirmed by immunoblotting analysis of cytoplasmic and nuclear fractions. Apo-7 mAb was able to immunoprecipitate endogenous A3G allowing to detect complexes with numerous proteins in activated-proliferating but not in resting CD4+ T cells. The results show for the first time the nuclear translocation of A3G in activated-proliferating CD4+ T cells.

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The APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like A3; A3) locus, found in human chromosome 22, comprises a cluster of seven genes (named A3A, A3B, A3C, A3D, A3F, A3G and A3H) encoding cytidine deaminases.1 The function of the A3 genes was initially unknown until a gene designated CEM15, which corresponded to A3G, was independently identified as a potent inhibitor of HIV-1 lacking Virion infectivity factor.2 Cytidine deaminases generate mutations in RNA and/or DNA by converting (deoxy) cytidine (C) to (deoxy)uridine (U).3,4 In humans, other members of the cytidine deaminase superfamily are:3,4 (i) APOBEC1, the founder of this superfamily and considered as a paradigm, which edits apolipoprotein B mRNA; (ii) activation-induced deaminase (AID), a germinal center B-cell-specific component essential for class-switch recombination and somatic hypermutation of rearranged immunoglobulin (Ig) variable gene segments; and (iii) APOBEC2 and APOBEC4, whose functions remain largely unknown.3,4

Although activated-proliferating CD4+ T cells are the main target cell for HIV-1 replication, blood resting CD4+ T-lymphocytes are refractory.5–7 Similarly, blood monocytes are also refractory, whereas once differentiated into macrophages are highly permissive for HIV-1 replication.6 In resting CD4+ T cells and monocytes, A3G was found to be expressed as a non-complexed sole component, in a so-called
low-molecular-mass (LMM) form, whereas after the activation of CD4+ T cells and the differentiation of monocytes into macrophages, it was recruited in a high-molecular-mass (HMM) form, where it bound to RNA together with multiple RNA-binding proteins, forming a ribonucleoprotein complex devoid of anti-viral activity.5,10 While initial investigations pointed out that A3G in its LMM form accounted for the resistance of resting CD4+ T cells and monocytes for the HIV replication,11 it was later on demonstrated that this is not the case, and a retraction was published.12 Additionally, two independent studies concluded that A3G is unlikely to have a role in the HIV-restrictive phenotype of quiescent T-lymphocytes.13,14

Immature (im) myeloid dendritic cell (mDCs) patrol mucosal territories to detect invader pathogens,15 and are among the earliest cells to encounter the HIV-1 virions transmitted through sexual contact. HIV-1 exploits the antigen-presenting cell functions of DCs to be transferred to CD4+ T cells,16 through the so-called virological synapse.19 This transfer, known as trans-infection, constitutes a mechanism of HIV-1 spreading and occurs even in the absence of productive infection of DCs.17,18,20 In fact, imDCs are highly resistant to productive HIV-1 infection, and mature DCs (mDCs) are even more resistant than imDCs, although, intriguingly, they are much more effective than imDCs to transfer HIV-1 to CD4+ T cells in the absence of their productive HIV infection.17,20,22–24 Importantly, based on RNA-silencing experiments the resistance of DCs to productive HIV-1 infection was found to depend on the expression of LMM A3G/A3F.24 A3G RNA-silencing experiments performed by others in DCs confirmed the important role of A3G to restrict HIV infection in these cells,25 a situation contrasting with the discrepancies indicated above, about A3G RNA-silencing experiments in resting CD4+ T cells. It should be noted that while A3G, A3F24 and A3A26 constitute HIV-1-restricting factors of DCs and other myeloid cells, SAMHD127 is the main HIV-1 restriction factor in myeloid cells.

The present work was aimed at investigating the differences regarding the subcellular localization of A3G in resting and activated CD4+ T-lymphocytes and in imDCs and mDCs. Although a plethora of studies exist on the interaction of A3G protein and HIV-1 using transfected cell lines,11,28,29 little attention has been paid to the activation-mediated regulation and subcellular localization of physiologic endogenous A3G protein in normal primary CD4+ T cells and imDCs and mDCs, a situation likely due to the lack of appropriate antibodies (Abs) to study native endogenous A3G protein. Here, we generated a mouse monoclonal Ab (mAb), designated Apo-7, that is unique in its ability to bind to and to immunoprecipitate endogenous A3G. The use of this mAb, in conjunction with other conventional procedures, allowed us (i) to demonstrate for the first time that A3G protein is cytoplasmic in resting CD4+ T cells and imDCs, whereas it largely translocates to the nucleus after CD4+ T-cell activation, but remains cytoplasmic after DC maturation; (ii) to easily detect that A3G was a non-complexed component in resting T cells, imDCs and mDCs, whereas it appeared complexed with numerous cell proteins in activated-proliferating CD4+ T cells. The translocation of A3G to the nucleus of activated-proliferating normal CD4+ T cells suggests that A3G has physiological functions in the nucleus of these cells.

RESULTS

Generation of anti-A3G mAbs

The lack of appropriate Abs against A3G is an important drawback in the analysis of the regulation of endogenous A3G protein and its subcellular location in non-transfected primary cells. Thus, we first tried to develop mouse hybridomas secreting anti-A3G mAbs able to bind endogenous A3G. As an immunizing antigen, we used rhuA3G-GST (glutathione-S-transferase) produced in insect Sf9 cells.20 Hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for production of mouse IgGs reactive to plate-bound rhuA3G-GST and non-reactive to plate-bound GST. Two hybridomas, designated Apo-5 and Apo-7, produced mAbs fulfilling these criteria and both were of IgG1κ isotype. The ELISA reactivity of Apo-7 mAb with plate-bound recombinant A3G (rA3G) was clearly much higher compared with that of Apo-5 mAb (Figure 1a). In contrast, when tested by western blot analysis, Apo-7

Figure 1 Characterization of Apo-5 and Apo-7 mAbs reactivity. (a) ELISA reactivity for a wide range of concentrations of Apo-5 and Apo-7 mAbs with plate-bound rhuA3G-GST protein. (b) Reactivity of Apo-5 (5 μg ml−1) and Apo-7 (5 μg ml−1) tested by western blot with 1 μg of rhuA3G-GST (A3G) and 1 μg of GST alone (GST); as a positive control, serum from rhuA3G-GST-immunized mice (pAb-A3G, 1:4000 diluted) was used. (c) Reactivity by western blot of Apo-5 and Apo-7 against rA3G (1 μg) and rA3F (1 μg); positive controls were anti-A3G rabbit pAb (α-A3G, 1:10 000 diluted) and anti-A3F rabbit pAb (α-A3F, 1:6000 diluted). Coomassie brilliant blue staining (Coomassie) was carried out to control protein load. In (a−c), data from a representative out of three independent experiments are shown. A full color version of this figure is available online at the Immunology and Cell Biology website.
mAb completely failed to react with both rA3G-GST and GST, whereas Apo-5 mAb reacted strongly with rA3G-GST, but not with GST alone (Figure 1b). A3G shares sequence homology with other members of the A3 locus, especially with A3F.\textsuperscript{3,11,28,30} We then examined by western blot the reactivity of Apo-5 and Apo-7 mAbs against \textit{Escherichia coli}-produced rA3G and rA3F. Anti-A3G and anti-A3F rabbit polyclonal Ab (pAb) were used as positive Ab controls. We found that Apo-7 mAb was unable to bind either rA3G or rA3F in western blot analysis (Figure 1c), whereas Apo-5 mAb showed just a weak binding to \textit{E. coli}-produced rA3G and lacked reactivity with rA3F (Figure 1c); this low reactivity of Apo-5 mAb with \textit{E. coli}-produced rA3G, compared with its high reactivity with Sf9-produced rA3G (Figure 1b), suggests that the epitope recognized by this mAb is related to the presence of a post-translational modification present in Sf9-produced A3G. The reactivity of Apo-7 mAb was further addressed by ELISA against rA3G, A3F and A3B (available through the NIH AIDS Reagent Program), which display high sequence homology.\textsuperscript{31} As shown in Figure 1s, Apo-7 mAb bound A3G, whereas it did not detectably bind A3F or A3B. Altogether, these data indicate that Apo-7 mAb recognizes a conformational epitope in native endogenous A3G, which is lost after denaturation as imposed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), whereas Apo-5 mAb recognizes a non-conformational epitope that is mainly expressed in denatured A3G. However, Apo-5 mAb failed to give clear results in western blot analysis of endogenous A3G in human cell lysates (data not shown), and therefore this mAb lacks interest to study A3G.

**Apo-7 mAb binds and immunoprecipitates endogenous native A3G**

As Apo-7 mAb recognizes a conformational epitope in non-denatured rA3G produced in Sf9 cells, it could also recognize native endogenous A3G using intracellular staining and immunoprecipitation procedures. We tested this possibility in the ‘non-permissive’ cell line CEM, which is known to constitutively express A3G, and in the ‘permissive’ CEM-subline, CEM-SS, which does not express or expresses very low amounts of A3G.\textsuperscript{2} We first confirmed the pattern of A3G expression in these cell lines at the mRNA level by real-time reverse transcription-polymerase chain reaction (RT-PCR) and at the protein level by western blot using rabbit anti-A3G pAb (Figures 2a and b). As A3G and A3F share a very similar sequence homology (62%; 90% in the promoter region)\textsuperscript{3,30} and are coordinately expressed,\textsuperscript{11,28,29} we also confirmed by western blot, with anti-A3F rabbit pAb, that A3F was also expressed in CEM cells, whereas it was nearly undetectable in CEM-SS cells (Figure 2b).

**Figure 2** Apo-7 mAb recognizes endogenous native A3G protein in CEM cells. (a) Relative levels of A3G mRNA expression in CEM and CEM-SS cells were quantified by real-time RT-PCR. Data represent the mean±s.d. of triplicates. *P<0.005 by two-tailed unpaired Student’s t-test. (b) Expression of A3G and A3F proteins analyzed by western blot in lysates (50 μg of protein per lane) of CEM and CEM-SS cells using anti-A3G (α-A3G) and anti-A3F (α-A3F) rabbit pAbs; as a control of protein loading per lane, a mouse anti-actin mAb (α-actin) was used to assess actin expression in CEM and CEM-SS cell lysates. (c) Immunoprecipitation of A3G from lysates of CEM or CEM-SS cell lines was carried out using Apo-7 mAb or with isotype-matched negative control IgG (IgG-Neg); immunoprecipitated material was analyzed by western blot (WB) by using rabbit anti-A3G pAb (α-A3G). (d) Reactivity of AlexaFluor488-conjugated Apo-7 mAb (solid black line) was assessed by flow cytometry in fixed and permeabilized CEM and CEM-SS cells. As a negative control, AlexaFluor488-conjugated isotype-matched negative control (Cl−, gray thin lines) was used. The percentage (%) of Apo-7 mAb-labeled cells (versus negative control) and the MFI value are indicated. In panels a–d, data from a representative out of three independent experiments are shown.
After these control experiments, we examined the binding of Apo-7 mAb to endogenous native A3G by immunoprecipitation in lysates of CEM and CEM-SS cells. The immunoprecipitated proteins were resolved by SDS-PAGE and revealed by western blot using rabbit anti-A3G pAb. As shown in Figure 2c, Apo-7 mAb clearly immunoprecipitated a protein band of ~46 kDa from CEM cells (Figure 2c), which was virtually absent in CEM-SS cells (Figure 2c). Notably, the Apo-7 mAb-immunoprecipitated protein completely failed to react with rabbit anti-A3F pAbs (data not shown), a result that was also found in human peripheral blood mononuclear cells (PBMCs) (see below).

We then tested by flow cytometry the capability of AlexaFluor488-conjugated Apo-7 mAb to bind endogenous native A3G protein in fixed and permeabilized CEM and CEM-SS cells. As depicted in Figure 2d, Apo-7 mAb bound most CEM cells (>90% of positive cells compared with an isotype-matched negative control), whereas it was hardly reactive with CEM-SS cells (~14% of positive cells); the mean fluorescence intensity (MFI) was 5.8 in CEM-SS cells versus 20.2 in CEM cells. The binding of Apo-5 mAb to endogenous native A3G was also examined, and, as expected, results from both CEM and CEM-SS cell lines (Figures 2c and d) were indistinguishable from that of isotype-matched negative control (data not shown). Taken together, these data indicate that the Apo-7 mAb constitutes an invaluable novel tool for the analysis of endogenous native A3G by either immunoprecipitation or cell staining procedures.

Activation of human CD4+ T cells upregulates A3G mRNA and protein expression

To address whether A3G expression is regulated during CD4+ T-cell activation, A3G mRNA and protein levels were investigated in PBMCs from healthy donors before and after 3 days of culture in the presence of phytohemagglutinin (PHA) or the T-cell superantigen, Staphylococcal enterotoxin A (SEA). The results (Figure 3a) show that activation of PBMCs with PHA or SEA involves a marked upregulation of A3G mRNA levels (respectively, 16- and 22-fold higher compared with mRNA levels in unstimulated PBMCs). Accordingly, A3G protein expression was also strongly increased after activation with PHA and SEA, as assessed by western blot of cell lysates using rabbit anti-A3G pAb (Figure 3b).

The expression of A3G protein in unstimulated and SEA-activated PBMCs was also analyzed by immunoprecipitation with the Apo-7 mAb. As found in CEM cells, Apo-7 mAb immunoprecipitated a protein band of ~46 kDa that was revealed by anti-A3G rabbit pAb, whereas it was fully unreactive with anti-A3F rabbit pAb, clearly...
demonstrating the lack of crossreactivity of Apo-7 mAb with A3F (Figure 3c). This increase in the immunoprecipitated material corroborated the strong activation-induced upregulation of A3G protein expression in PBMCs (Figure 3c).

We also evaluated the expression of endogenous native A3G protein in the CD4-gated population of resting and PHA- or SEA-activated PBMCs by cell staining and flow cytometry analysis using AlexaFluor488-conjugated Apo-7 mAb. The results (Figure 3d) show that the whole population of resting CD4+ T cells expresses A3G at low levels (MFI value of 29), whereas in activated CD4+ T cells, A3G expression was higher after PHA (MFI value of 245) or SEA (MFI value of 57) stimulation (Figure 3d). Interestingly, A3G mRNA levels were higher in SEA-treated compared with that in PHA-treated PBMCs (Figure 3a), whereas A3G protein levels in CD4+ T cells were higher in PHA-treated compared with that in SEA-treated lymphocytes (Figure 3d). According to this observation, we were wondering whether expression of A3G protein was upregulated with different kinetics in PBMCs when treated with PHA versus SEA. In addition, we wanted to know whether upregulation of A3G protein expression also occurred in other populations contained in PBMCs different from CD4+ T cells, such as the CD11c+ population. For this purpose, we stimulated PBMCs with PHA or SEA and analyzed A3G expression by flow cytometry at different time points in total PBMCs, and in gated CD4+ T cells and gated CD11c+ cells (Supplementary Figure S2). The results show that following PHA or SEA stimulation, A3G expression was upregulated not only in CD4+ T cells but also in the CD11c+ population and that the kinetics of this upregulation was similar in total PBMCs, in gated CD4+ T cells and in CD11c+ cells (Supplementary Figure S2). Accordingly, we next extended our interest to the analysis of A3G expression in DCs.

Upregulation of A3G expression following the MDDCs and blood myDCs and plasmacytoid DCs

To address potential changes in expression levels of A3G mRNA and protein in imDCs and mDCs, we used maturation of monocyte-derived DCs (MDDCs) before and after a 48-h culture with the proinflammatory maturation cocktail (IL-1β+IL-6+TNF-α+PGE2). The effects of each component of this cocktail were also analyzed. As shown in Figure 4a, the cocktail caused a great increase in the A3G mRNA levels. TNF-α (tumor necrosis factor-α) or PGE2 (prostaglandin E2) alone also induced a significant upregulation of A3G mRNA levels, whereas IL-1β (interleukin-1β) or IL-6 did not (Figure 4a). The expression of A3G protein levels assessed by western blot analysis using rabbit anti-A3G pAb were increased by the maturation cocktail: TNF-α alone also induced a considerable augmentation, whereas a modest increase was observed with IL-1β, IL-6 or PGE2 (Figure 4b).

The expression of endogenous native A3G protein was also evaluated by intracellular staining and flow cytometry analysis using AlexaFluor488-conjugated Apo-7 mAb in the CD11c+ gated population of MDDCs before and after 48-h culture with the maturation cocktail. Results clearly corroborated the increase of A3G protein expression after DC maturation (Figure 4c). Indeed, the MFI value was increased at least two times in mMDDCs when compared with imMDDCs (Figure 4c). Taken together, these results indicate that the proinflammatory maturation cocktail promotes an augmented expression of A3G mRNA and protein in MDDCs, with TNF-α being the major contributor.

To corroborate the above findings in more physiological DC populations, we analyzed the expression of A3G mRNA in highly purified (98–99%) blood-circulating myDCs and plasmacytoid DCs (pcDCs) after 2 days of cell culture in the absence or presence of maturation/activation stimuli, LPS+CD40L (lipopolysaccharide +CD40-ligand) for myDCs and IL-3+CD40L+CpG-ODN for pcDCs (see Methods). As shown in Figure 4d, unstimulated myDCs and pcDCs expressed very low A3G mRNA levels, which increased strongly after culture with maturation stimuli. These data demonstrate that DC maturation results in a highly enhanced expression of A3G mRNA not only in MDDCs matured with the proinflammatory cocktail but also in physiological blood-circulating myDCs and pcDCs matured with completely different stimuli.

Analysis of subcellular location of A3G protein in CD4+ T cells and monocyte-derived DCs

We also assessed the binding of Apo-7 mAb to endogenous A3G in fixed and permeabilized cells. The subcellular location of the protein was first assessed in CEM and CEM-SS cell lines. As expected, the binding of Apo-7 mAb to CEM-SS cells was undetectable even after 24 h of stimulation with PHA (Figure 5). By contrast, A3G was clearly detectable in unstimulated CEM cells, and the weak labeling was perinuclear with punctuate intensifications. Following PHA stimulation of CEM cells, A3G expression was highly increased and it appeared largely translocated to the nuclei (Figure 5).

After these experiments in cell lines, we analyzed the intracellular distribution of A3G protein by using Apo-7 mAb in primary CD4+ T cells from PBMCs of healthy donors before and after 3 days of PHA- or SEA-induced activation. As shown in Figure 5, we found in resting CD4+ T cells that A3G protein was found in the perinuclear compartment with punctuate intensifications. By contrast, in PHA- and SEA-activated CD4+ T cells, the A3G protein expression was strongly increased and a significant staining appeared in the nuclei (Figure 5).

The subcellular distribution of A3G using Apo-7 mAb and confocal microscopy was also investigated in monocyte-derived DCs from healthy subjects, before and after 2 days of cocktail-induced maturation. As it occurred in resting CD4+ T cells, endogenous A3G was perinuclear in imMDDCs, whereas in mMDDCs the expression of A3G increased strongly but, in sharp contrast to results in activated CD4+ T cells, remained retained in the cytoplasm (see Supplementary Figure S3).

As numerous studies on the subcellular A3G localization performed with transfected cell lines have reported that A3G is cytoplasmic, we wanted to confirm the findings using a completely distinct and semiquantitative procedure. For such purpose, the expression of A3G protein was determined in isolated nuclear and cytoplasmic fractions from resting and activated T cells, and from imDCs and mDCs. Western blot analysis using rabbit anti-A3G pAbs confirmed that the activation of T cells involves a concomitant translocation of A3G protein from the cytoplasmic to the nuclear fraction (Figure 6a), whereas the proportion of A3G in the cytoplasmic and nuclear fractions of MDDCs, before and after their maturation, remained similar (Figure 6b). These data corroborate the above results of subcellular distribution assessed by confocal microscopy using Apo-7 mAb (Figure 5 and Supplementary Figure S3), and demonstrate for the first time that the increased A3G expression that occurs following the activation of CD4+ T-lymphocytes, and the maturation of DCs, involves its translocation to the nucleus in the case of activated T cells but not in the case of mDCs.

Analysis of LMM and HMM forms of endogenous A3G from T cells and MDDCs by immunoprecipitation with Apo-7 mAb

A3G has been found to exist in an LMM form in resting CD4+ T cells, and as an HMM ribonucleoprotein complex in mitogen-activated CD4+ T cells. In the HMM ribonucleoprotein complex, A3G is
found associated with numerous RNA-binding host cell proteins, whereas in the LMM form it is found as a unique component. In MDDCs, A3G was only found to be expressed in an LMM form. Apo-7 was used to better understand the nature of LMM and HMM forms. Immunoprecipitates from imMDDCs and mMDDCs and from resting and activated T cells were separated by SDS-PAGE followed by detection with silver staining. As shown in Figure 7a, A3G expressed in resting PBMCs was immunoprecipitated as a unique protein band of ~46 kDa. The same occurred in both imMDDCs and mMDDCs (Figure 7b). By contrast, in PHA-treated PBMCs, A3G was co-immunoprecipitated with many other different proteins (Figure 7a). These results suggest that cytoplasmic A3G is expressed as a single band and is not subject to proteolytic degradation in resting PBMCs.
largely monomeric, whereas hetero-oligomeric complexes occur upon translocation to the nucleus. As evidenced by a band appearing at \( \sim 100-105 \) kDa, it is also worth noting that A3G seems to co-immunoprecipitate in mMDDC with one or more other proteins, whose identification would need further investigation.

**DISCUSSION**

Upregulation of A3G has been reported in monocytes and macrophages treated with interferon-\( \alpha \).42,43 A3G expression has been also analyzed in primary T-lymphocytes, macrophages and MDDCs,41,44 in plasmacytid-circulating DCs,45 in poly I:C-matured MDDCs,46 in primary hematopoietic cell subsets47,48 and in CD4\(^+\) T-cell helper-1 (Th1) and Th2 subsets.49 None of these studies analyzed the subcellular distribution of A3G protein. Similarly, none of these studies assessed the individual cell-based expression of A3G protein by flow cytometry as performed here using the Apo-7 mAb. Apo-7 mAb likely recognizes a nonlinear conformational epitope on A3G, and therefore is unable to detect denaturated A3G by western blot.
A3G expression in T cells and dendritic cells

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Figure 7. Endogenous A3G immunoprecipitated by the Apo-7 mAb is associated with many other proteins in activated T cells, whereas it appears as a unique monomeric protein in resting T cells and in imMDDC and mMDDCs. Lysates were obtained (a) from PBMCs before (Resting) and after 72 h of culture with PHA, and (b) from imMDDCs and after their 48 h maturation with the standard cocktail (mMDDCs), and subjected to immunoprecipitation with anti-A3G Apo-7 mAb or isotype-matched negative control (IgG). Immunoprecipitated proteins were analyzed by SDS-PAGE followed by silver staining. Data from a representative out of three independent experiments with cells from different healthy donors are shown. The arrow on the right indicates the molecular mass of 46 kDa.

After SDS-PAGE. In contrast, it is able to bind endogenous native A3G in cell staining and immunoprecipitation procedures. This antibody is a valuable new tool as commercially available pAbs are appropriate for western blot analysis but not so useful for flow cytometry.

This study takes advantage of the development of a novel anti-A3G monoclonal antibody and shows that A3G in both resting CD4+ T cells and imDCs is modestly expressed, is monomeric and display a perinuclear location, whereas is strongly upregulated in CD4+ T-lymphocytes after their mitogenic activation and in mDCs. This similarity in the A3G upregulation following the activation of CD4+ T cells and the maturation of DCs is coupled with two sharp differences: in activated-proliferating CD4+ T cells, A3G is largely translocated to the nucleus and appears complexed with other nuclear proteins, whereas in mDCs, A3G remains in the cytoplasm as a unique, non-complexed form. It should be noted that the differential subcellular distribution of A3G between resting and activated T cells was confirmed by a fully different and semiquantitative procedure such as western blot in the cytoplasmic and nuclear fractions using a rabbit anti-A3G pAb. To our knowledge, no other studies have analyzed the subcellular localization of endogenous A3G in resting and activated T cells and in imDCs and mDCs. Thus, our results demonstrate for the first time that mitogenic activation of primary CD4+ T cells leads to A3G translocation to the nucleus where it interacts with other proteins. These results explain from a biochemical perspective the previously reported LMM and HMM forms of the protein.

The A3 members show a high degree of homology. Thus, Apo-7 mAb, apart from recognizing A3G, could also crossreact with other A3 proteins. In fact, rabbit anti-A3G pAbs may crossreact with A3A, although this is not the case of rabbit anti-A3F pAbs. We experimentally excluded the crossreactivity of Apo-7 mAb with A3F protein, the A3 member with the highest similarity with A3G (65%; 90% in the promoter region) and also with A3B (see Figure 3c and Supplementary Figure S1). On the other hand, whereas flow cytometry analysis revealed a clear intracellular binding of Apo-7 mAb to CEM cells (MFI 20.2 and positivity >90%), just a weak binding of Apo-7 mAb was detected in CEM-SS cells (MFI 5.4 and 14% positivity). This differential pattern of Apo-7 mAb binding seems fully consistent with the differential expression of A3G and A3F in these cell lines. Indeed, compared with CEM line, A3G protein expression is very low but not absent in CEM-SS, and A3F protein is undetectable (refs 2,4,7,48 and Figure 2). Importantly, A3A, A3C and A3H have a molecular mass that is approximately half (~20–22 kDa) of that of A3B, A3D, A3F and A3G (~46 kDa). As A3 protein recognized by Apo-7 mAb in immunoprecipitation experiments corresponds to a unique band of ~46 kDa, one may conclude that this antibody does not crossreact with A3A, A3C and A3H proteins. Another possibility would be that Apo-7 mAb could have some degree of crossreactivity with A3A, A3C and A3H proteins but their expression would be too low to be detected; nevertheless, this possibility seems unlikely because: (i) although A3A mRNA is undetectable in both CEM and CEM-SS lines, it is considerably high in PBMCs, mainly due to its expression in monocytes; (ii) A3C mRNA is highly expressed in CEM cells, as well as in PBMCs and activated T cells; and (iii) although A3H mRNA content is very low in both CEM and CEM-SS, it reaches a significant expression in activated T cells. Regarding the A3B, it is the only family member of exclusive nuclear localization. Because Apo-7 mAb showed no nuclear staining in unstimulated CEM cells (Figure 5), it is not likely that this mAb recognizes A3B, a finding clearly consistent with the lack of ELISA reactivity of Apo-7 mAb with rA3B (Supplementary Figure S1). Overall, these data strongly suggest that Apo-7 mAb recognizes endogenous A3G with high specificity with respect to the other A3 family members.

Studies aimed at analyzing the potential relationship between the rate of HIV-1 disease progression rate and A3G mRNA levels have provided controversial results (see also Albin and Harris and references therein). Association of A3G expression with resistance to
HIV-1 infection is also a controversial issue. The differential activation state of PBMCs leading to gross interpatient differences in A3G expression may be a likely confounder factor. Apo-7 mAb may be useful to minimize this confounder factor by quantification of the A3G protein expression in the different T-cell subsets by multichromatic flow cytometry at a single cell level.

There is evidence indicating that the A3 gene cluster has been subjected to strong positive selection during the primate evolution, suggesting that this gene cluster has evolved to counteract the genomic instability imposed by endogenous retrotransposons in primates (see Chiu and Greene and Arias and references therein). In agreement with this notion, apart from inhibiting HIV-1, A3G also inhibits other retroviruses, including mouse and yeast endogenous retroviruses, and also hepatitis B virus, a DNA virus that requires a retrotranscription RNA intermediate (see refs 4, 11, 28, 29, 63 and references therein). Moreover, several A3 members inhibit long interspaced retrotransposons to be retrotranscribed in the nucleus (see Arias and references therein). Regarding the nuclear A3G translocation observed here in activated normal CD4+ T cells, it is noteworthy that A3G can reach a superantigen expression in T cells and dendritic cells.

Methods
Ethic statement
This study received the approval of the Committee of Ethics and Clinical Investigation of our Institution (Hospital Clinic de Barcelona, Barcelona, Spain). All the subjects participating in the study were voluntary healthy donors and gave their informed written consent. All animal procedures were carried out in accordance with the European legislation on the use and care of laboratory animals (86/609/EEC).

Generation of anti-A3G mAbs
BALB/c mice received three intraperitoneal injections, at days 0, 22 and 37, of 10 μg of A3G-GST fusion protein recombinant human A3G-GST (rhuA3G-GST) expressed in insect Sf9 cells, emulsified in CFA for the first injection and in IFA for the two boosting injections. Only mice with detectable serum anti-A3G Abs were used for somatic hybridization of their splenocytes with NS1 plasmacytoma cells. Anti-A3G Abs in mice sera and hybridoma culture supernatants were detected by ELISA with microwell plates coated with 100 μl per well of phosphate-buffered saline containing 1 μg ml⁻¹ of S99-produced rhuA3G-GST; the reactivity with plate-bound GST was also assessed in parallel. Mouse IgG reacting with plate-bound antigens was detected by horseradish peroxidase-conjugated goat anti-mouse-IgG Abs (Dako, Glostrup, Denmark). Two antibodies reacting against A3G were successfully isolated: Apo-5 and Apo-7. The ELISA reactivity of Apo-7 mAb was also assessed against plate-bound rA3G, A3F and A3B, obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH), from Immunodiagnostics (Woburn, MA, USA), respective catalog numbers: 10068, 11097 and 11099. Proteins were used to coat ELISA plates at a final concentration of 5 μg ml⁻¹ in phosphate-buffered saline. The positive controls used to detect those proteins were: anti-human A3B rabbit monoclonal 5210-87-13 (catalog number 12397), known to crossreact with A3A and A3G, and A3F chicken antisera (catalog number 11425), both donated by Dr Reuben Harris (also obtained through the NIH AIDS Reagent Program). Plate-bound recombinant SIVmac239 gp140 protein (Immune Technology Corp., New York, NY, USA) was used as a negative control and was detected with serum from an SIV (Simian immunodeficiency virus)-infected monkey. The secondary antibodies used were goat anti-mouse IgG1 (107-05), goat anti-rabbit (4049-05) and goat anti-chicken (6100-05), all three obtained from SouthernBiotech (Birmingham, AL, USA), and goat anti-rhesus (sc2453), purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell lines and PBMCs
CEM and CEM-SS cell lines, donated by NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH), negative for bacteria, mycoplasma and fungi were cultured in RPMI-1640 containing 10% fetal bovine serum. When indicated, these cell lines were activated overnight with 10 μg ml⁻¹ of PHA (from Sigma, St Louis, MO, USA). PBMCs from healthy volunteers were isolated immediately after venous blood extraction using standard Ficoll gradient centrifugation. Fresh PBMCs, resuspended (1x10⁶ cells per ml) in serum-free X-VIVO-10 (Bio-Whittaker, Walkersville, MD, USA) were cultured for 3 days in the absence or presence of mitogenic stimuli such as 10 ng ml⁻¹ of the T-cell superantigen SEA or 10 ng ml⁻¹ of PHA (both from Sigma).

Generation of imMDDCs and mMDDCs
Monocytes from fresh PBMC of healthy volunteers were isolated by adherence to plastic cell culture flasks as already reported and were differentiated into imMDDCs in a 5-day culture in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (final concentration 1000 U ml⁻¹ each; Prospek-Tany Technogenie Ltd, Rehovot, Israel); these cytokines were added at days 0, 3 and 5. Maturation of MDDCs was achieved with the standard cocktail containing TNF-α (1000 U ml⁻¹), IL-1β (10 ng ml⁻¹), IL-6 (1000 U ml⁻¹) (from Stratham Biotec AG, Hamburg, Germany) and PGE2 (1 μg ml⁻¹; from Sigma); this cocktail was added at day 5, and the culture prolonged for two additional days. Purity and immunophenotype of imMDDCs and mMDDCs were assessed by flow cytometry as reported (see Table 1).

Table 1 Immunophenotype of immature and mature MDDCs

<table>
<thead>
<tr>
<th>MDDCs⁹</th>
<th>CD14</th>
<th>HLA-DR</th>
<th>CD11c</th>
<th>CD209</th>
<th>CD86</th>
<th>CD80</th>
<th>CD83</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>≤3%</td>
<td>&gt;99%</td>
<td>&gt;90%</td>
<td>&gt;80%</td>
<td>&gt;80%</td>
<td>&gt;2%</td>
<td>&gt;2%</td>
</tr>
<tr>
<td>Mature</td>
<td>≤3%</td>
<td>&gt;99%</td>
<td>&gt;95%</td>
<td>≤45%</td>
<td>&gt;99%</td>
<td>&gt;80%</td>
<td>&gt;80%</td>
</tr>
</tbody>
</table>

Abbreviations: FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; mAb, monoclonal antibodies; MDDC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin.

*Fresh PBMCs were resuspended (3x10⁶ cells per ml) in serum-free culture medium (X-VIVO-15) supplemented with 1% of heat-inactivated human AB serum, and monocytes were isolated by adherence (2 h at 37 °C) to plastic cell culture flasks. After washing (4x) with 37 °C prewarmed X-VIVO-10, adherent cells (≥95% CD14⁺) were differentiated into immature MDDCs by a 5-day culture in X-VIVO-15 containing IL-4 and GM-CSF (1000 U ml⁻¹ each); these cytokines were added at days 0, 3 and 5. To obtain mature MDDCs, the standard cocktail (see the text) was added at day 5 and the culture prolonged for two additional days. Cells were analyzed by two-color flow cytometry using PE-conjugated mAbs recognizing CD14, CD11c, CD209, CD86, CD80, CD83 and FITC-conjugated anti-CD11c mAb compared with isotype-matched PE- and FITC-conjugated negative control mAbs (all mAbs from BD Biosciences).

Contaminating lymphocytes were found to be ≤3% as assessed by flow cytometry analysis with fluorochrome-conjugated anti-CD3, anti-CD19 and anti-CD66 mAbs. Values represent % of positive cells for each marker in a representative experiment out of five from different healthy individuals.
Isolation and maturation/activation of circulating blood myDCs
and pDCs
Isolation of myDCs and pDCs from healthy-blood buffy coats was performed using the Blood Dendritic Cell Isolation Kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Purity for pDCs and myDCs yielded ≥98%. To induce maturation/activation of DCs, myDCs were incubated for 42 h with 1 μg ml⁻¹ lipopolysaccharide (Sigma) and 125 ng ml⁻¹ of CD40L (Bender Medystems, Vienna, Austria), whereas pDCs were exposed to 125 ng ml⁻¹ CD40L, 25 ng ml⁻¹ IL-3 (Peprotech, London, UK) and 5 μg ml⁻¹ endotoxin-free Cpg (Invitrogen Corporation, Paisley, Scotland) as described.²⁴

Western blot
Cell lysates (50 μg per lane), obtained in ice-cold lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM NaVO₃, 20 mM Tris-Cl, pH 7.5; with complete protease inhibitor cocktail from Sigma), or Sf9-produced rhuA3G-GST protein or E. coli-produced rhuA3G or A3F proteins (1 μg per lane), from NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH), as indicated in the Results section, were resolved by SDS-PAGE and transferred to PVDF membranes (Sigma), which were then incubated with the primary Abs as indicated in the Results section and revealed with secondary horseradish peroxidase-conjugated goat pAb (1:1000) against mouse or rabbit IgG (Pierce, Rockford, IL, USA). Primary rabbit pAbs developed against C-terminal peptide of human A3G (catalog number 10201, donated by Dr Jaisri Lingappa), and A3F proteins were obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Membranes were stripped and reblotted with anti-actin mouse mAb (1:2000; from Sigma) or with anti-human β2m rabbit pAb (from Sigma), and then incubated with secondary horseradish peroxidase-conjugated goat pAb as above.

Immunoprecipitation with Apo-7 mAb
Cells lysates, obtained as above, were treated with 5 μg ml⁻¹ of Apo-7 or isotype-matched control (BD Biosciences, Madrid, Spain) and immunoprecipitated using the Protein G Immunoprecipitation Kit (Sigma). The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Silver Staining Kit (Bio-Rad Laboratories, Barcelona, Spain) or by immunodetection by western blot analysis using rabbit anti-A3G and anti-A3F pAbs as indicated above.

Isolation of cytoplasmic and nuclear fractions
Cells were treated with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). A3G was immunodetected by western blot using the rabbit anti-A3G pAb. Relative quantification of A3G from nuclear and cytoplasmic fractions was performed by densitometric analysis using the UN-SCAN-IT gel software (Silk Scientific, Inc. Orem, UT, USA). The percentage of A3G distribution was calculated as: (pixels in a fraction) × 10⁰/(pixels in nucleus + pixels in cytosol). Western blots were carried out in parallel to assess the purity of cytoplasmic and nuclear fractions using mouse anti-β-tubulin-mAb (1:2000; Sigma) or rabbit anti-histone H3pAb (1:10 000; Sigma), respectively.

Flow cytometry analysis
The purity and immunophenotype of immature and mature monocyte-derived DCs were assessed by flow cytometry analysis (Table 1) using commercially available mAbs (BD Biosciences) as already reported.²²,²³ To detect endogenous A3G with Apo-7 mAb, permeabilized cells (Cytofix and Cytoperm Kit; BD Biosciences) were incubated at 4 °C for 30 min with AlexaFluor488-conjugated Apo-7 mAb (10 μg ml⁻¹, final concentration); before permeabilization, cells were cell surface stained with fluorochrome-conjugated anti-CD4, anti-CD3, anti-CD8 and anti-CD11c mAbs. Conjugation of purified Apo-7 with Alexa-Fluor488 was performed with the labeling kit from Molecular Probes (Invitrogen, Grand Island, NY, USA).

Immunostaining for confocal microscopy analysis
Cells (4 × 10⁶ cells in 50 μl phosphate-buffered saline) were plated on poly-l-lysine (from Sigma)-coated coverslips, were fixed and permeabilized as above and incubated with blocking buffer and incubated with 10 μg ml⁻¹ of purified Apo-7 mAb or mouse IgG as a negative control. After washing, coverslips were incubated with biotin-conjugated goat anti-mouse IgG1 (1:1000 diluted; Santa Cruz Biotechnology), washed again and incubated with TRITC-conjugated streptavidin (1:100 diluted; Serotec, Puchheim, Germany). Thereafter, coverslips were washed and incubated with phosphate-buffered saline containing 5% heat-inactivated mouse serum. All these incubations were carried out at room temperature for 30 min. Subsequently, coverslips were incubated at 4 °C for 30 min with either FITC-conjugated anti-human-CD40-mAb (1:10 diluted; Serotec) for staining MDDCs or with FITC-conjugated anti-human-CD4-mAb (1:10 diluted; BD Biosciences) for staining PBMCs during the last 5 min of these incubations, Hoechst stain (1:10 000 diluted) was added. Washed coverslips were mounted with immunoFluor mounting medium (ICN Biomedica Inc., Irvine, CA, USA). Confocal images were acquired using a Leica TCS SI laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with argon and HeNe lasers attached to a Leica DMIRE2 inverted microscope (Leica, Wetzlar, Germany). Image assembly and treatment were performed using the Image Processing Leica Confocal Software (Leica).

Relative mRNA expression by real-time PCR
Total RNA was extracted from cells by using TRIzol reagent (Sigma). Aliquots of 1 μg total RNA, 0.5 μg of oligo-(dT) (Sigma) and 2 μl dNTPs (mix, 5 μM each; Sigma) were dissolved in sterile RNase-free water, in a volume of 12 μl and heat denatured at 65 °C for 5 min and cooled to 4 °C. Four microliters of First-Strand Buffer 5 x (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3; Invitrogen), 2 μl of 100 mM dithiothreitol (Invitrogen) and 1 μl of RNase inhibitor (RNase OUT; Invitrogen) were added and incubated at 42 °C for 2 min. Subsequently, 1 μl (200 U) of SuperScript II RT (Invitrogen) was added and cDNA synthesis performed at 42 °C for 50 min. The reaction was completed by further incubation at 70 °C for 15 min. The cDNAs were amplified using a LightCycler FastStart DNA Master²²,²³ SYBR Green-I Kit (Roche, Penzberg, Germany). Each reaction was carried out with 2 μl of cDNA, 4 μl of 5 x Master-Mix (FastStart DNA Master²²,²³ SYBR Green-I), 0.5 μM of each primer and sterile RNase-free water at a final volume of 20 μl. Real-time PCR were carried out for as described in Table S1 by 35 cycles using the LightCycler (Roche) (see Supplementary Table S1). To calculate relative levels of A3G mRNA, β2-microglobulin was used as housekeeping gene, except for CEM/CEM-SS cell lines, which express barely detectable levels of β2-microglobulin mRNA (data not shown): GAPDH mRNA instead of β2-microglobulin mRNA was used with these cell lines. Relative mRNA levels were calculated with the formula = 2 − (Ct of A3G − Ct of housekeeping) × 10⁻²⁷. Direct sequencing of amplified A3G cDNA was identical to that found in gene databases for human A3G.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Author contributions: Conceived the research: TG, NN; designed experiments: HO, TG; performed experiments: HO, RP, JMM-N, MN-G, NC, MR-G, CP, CG; analyzed data: HO, RP, JMM-N, MP, FG, JMM, CP, RF, JMG, TG; contributed reagents/materials/analysis tools: FB, NN; wrote the paper: HO, RP, JMM-N, RF, NN, TG.

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)