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Anti-HIV-1 antibody-dependent cellular cytotoxicity mediated by hyperimmune bovine colostrum IgG

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Antibodies with antibody-dependent cellular cytotoxicity (ADCC) activity play an important role in protection against HIV-1 infection, but generating sufficient amounts of antibodies to study their protective efficacy is difficult. HIV-specific IgG can be easily and inexpensively produced in large quantities using bovine colostrum. We previously vaccinated cows with HIV-1 envelope gp140 and elicited high titers of anti-gp140-binding IgG in colostrum. In the present study, we determined whether bovine antibodies would also demonstrate specific cytotoxic activity. We found that bovine IgG bind to Fcγ-receptors (FcγRs) on human neutrophils, monocytes, and NK cells in a dose-dependent manner. Antibody-dependent killing was observed in the presence of anti-HIV-1 colostrum IgG but not nonimmune colostrum IgG. Killing was dependent on Fc and FcγR interaction since ADDC activity was not seen with $F(ab')_2$ fragments. ADCC activity was primarily mediated by $CD14^+$ monocytes with FcγRIIa (CD32a) as the major receptor responsible for monocyte-mediated ADCC in response to bovine IgG. In conclusion, we demonstrate that bovine anti-HIV colostrum IgG have robust HIV-1-specific ADCC activity and therefore offer a useful source of antibodies able to provide a rapid and potent response against HIV-1 infection. This could assist the development of novel Ab-mediated approaches for prevention of HIV-1 transmission.

Keywords: Antibody-dependent cellular cytotoxicity (ADCC) · bovine hyperimmune colostrum · Fcγ receptor · HIV

Introduction

A vaccine or immunotherapy against the human immunodeficiency virus type 1 (HIV-1) is urgently needed. Neutralizing antibodies (NAbs) are usually the most efficient mode of antibody (Ab)-mediated defense against most viral infections. An enormous effort has been put into investigating NAb responses to HIV-1 and their epitope specificity, potency, and breadth. The broadly (b)NAbs investigated to date target primarily the envelope protein of HIV-1 [1]. Neutralizing activity is associated with long-term nonprogression [2] and can provide sterile protection after passive transfer of bNAbs, like b12, 2G12, or 2F5 in the macaque

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model [3, 4], and drives the generation of neutralization escape virus variants in vivo [5]. However, despite intensive efforts, no HIV-1 vaccines have reliably elicited bNAbs thus far.

Virus-specific binding but nonneutralizing Abs can also have substantial impact on clearing virus particles or virus-infected cells [6, 7]. Specifically, Abs that mediate antibody-dependent cellular cytotoxicity (ADCC) activity are a current focus of HIV-1 vaccine research. ADCC is initiated by Fc receptors (FcRs) on immune cells recognizing and binding IgG (IgG1 or IgG3) and IgA [8] bound to infected cells. FcR-bearing effector cells include innate immune cells such as NK cells, monocytes, and neutrophils. FcγRI (CD64), which is expressed on monocytes, can bind monomeric IgG with high affinity and therefore, given the high concentration of serum IgG, is thought to be saturated under physiological conditions. In contrast, efficient binding of low-affinity receptors FcγRIIa/b, (CD32a/b), and FcγRIIIa/b (CD16a/b) to monomeric IgG under physiological conditions requires the formation of immune complexes like IgG-opsonized target cells [9].

Considerable evidence supports a role for ADCC activity in the control of HIV-1 infection with a positive impact on disease progression [10–12]. In the context of vaccination, ADCC-Abs correlate with protection in animal models of HIV-1 infection [13]. The induction of protective immunity despite the lack of the induction of neutralizing Abs, which was observed in the recent partially successful HIV-1 vaccination clinical trial RV144 [14], suggests that nonneutralizing Abs with potential ADCC activity may play an active role [15]. Hessel et al. demonstrated the importance of IgG Fc-domain interaction with FcγR [16]. They showed that Fc-mediated effector function is essential for the protective efficacy of the b12 Nab, since mutation of the Fc portion substantially reduced the protective capacity of the NAbs. Further characterization of the bNabs 2F5, b12, and 2G12 confirmed their potent ADCC activity in vitro [17, 18]. Thus, investigation into ADCC-mediated mechanisms during HIV-1 is warranted.

Only a few anti-HIV-1 monoclonal Abs are described with ADCC activity in the absence of neutralization function [19]. The generation of large amounts of such Abs would be useful to either (i) study ADCC and dissect the specificities and epitopes of particularly useful ADCC Abs to enable the design of improved HIV-1 vaccines or (ii) assess the ability of these Abs to protect against mucosal HIV-1 infection if used in new prevention measurements such as Ab-based microbicides.

One novel method to generate large amounts of anti-HIV-1 Abs is to immunize pregnant cows with gp140 envelope oligomers and collect the colostrum (first milk) after birth. Bovine colostrum contains approximately 50 mg/mL of IgG (mainly IgG1) and 4 mg/mL of IgA [20]. The prophylactic potential of bovine colostrum has been shown for other pathogens such as those causing bacterial diarrhea [21]. Little is known about whether bovine Abs could also trigger cellular effector functions in humans. There is some evidence in the early studies suggesting that bovine IgG can bind to human B cells and monocytes [22], but bovine antibodies have not been assessed for their ability to mediate ADCC against HIV-1. The aim of our study was to investigate whether bovine colostrum IgG bind efficiently to FcγR on human blood cells and whether this binding could elicit specific ADCC.

Results

Bovine IgG bind to Fcγ receptors on human immune cells

We recently observed that cows vaccinated with gp140 oligomers before and after conception developed high IgG gp140 AD8 binding titers resulting in endpoint concentrations of 1.56 μg/μL for purified colostrum IgG. Polyclonal anti-HIV-1 bovine colostrum IgG from two cows also had broad neutralizing activity in vitro but their ability to promote ADCC activity was not determined [23]. To first determine the binding ability of bovine IgG to FcγR, we incubated washed human whole blood with bovine IgG and quantified bound IgG on various cell types with a fluorescently labeled $F(ab)_2$-specific anti-bovine IgG Ab. Bovine IgG bound to the greatest extent to FcγR expressed on neutrophils and $CD3^-CD14^+$ monocytes (Fig. 1A). Binding was also detected on $CD3^-CD56^{dim}$ NK cells to a lesser extent. Only background levels of binding were observed on $CD3^+$ T cells, consistent with the fact that they do not express FcγRs on their surface. Binding of bovine IgG to the FcγR on the respective cell subsets was dose dependent. Binding was saturated at a concentration of 0.1 mg/mL bovine IgG for neutrophils and monocytes but binding on NK cells was not saturated at concentrations up to 0.4 mg/mL (Fig. 1B). Binding to monocytes and neutrophils fit a simple one-site binding model with half-max concentration of 0.022 mg/mL (monocytes) and 0.0028 mg/mL (neutrophils) for bovine IgG. Binding of bovine IgG could be largely inhibited by preincubation with a mix of anti-CD16/32/64 blocking antibodies (Fig. 1C).

Bovine IgG specific to gp140 mediates killing of HIV-1 target cells as measured by the RFADCC assay

To determine the ability of bovine IgG to promote ADCC, we measured killing of gp140-coated ($gp140^+$) and gp140-uncoated ($gp140^-$) fluorescently labeled CEM.NKr-CCR5 target cells (targets) by PBMCs in the presence of bovine IgG. Loss of membrane integrity in the target cell during ADCC in this assay is indicated by the loss of intracellular CFSE and retention of the membrane dye PKH26 ($CFSE^-PKH26^+$) [24]. Serum of an HIV-1-infected patient with known ADCC activity [25] (HIV-immune serum) was used as a positive control to measure killing of target cells using PBMCs prepared from healthy donors as a source of effector cells (Fig. 2A). $PKH26^+$ cells were gated on according to Gomez-Roman et al. [24]. Target cells were not killed in the absence of IgG independent on gp140 coating (2A, left panel). No loss of CSFE was seen in targets incubated in the absence of effector PBMCs. Opsonization with serum from HIV uninfected donors did not induce killing (2A, middle panels). In contrast, opsonization of target cells with HIV-immune serum induced killing of $gp140^+$

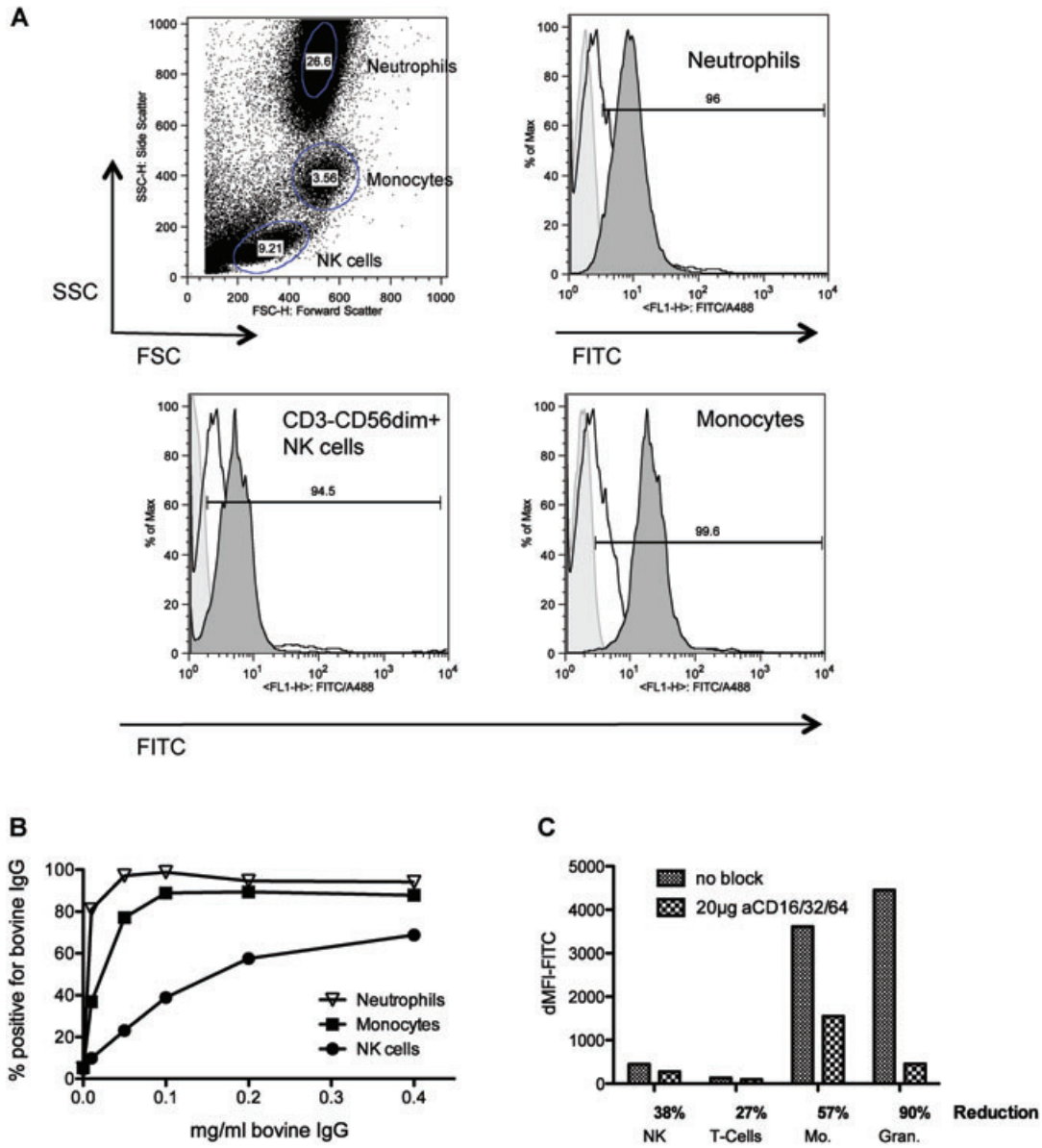

Figure 1. Binding of purified bovine IgG to human FcγR. Washed whole blood was gated for white blood cells based on FSC/SSC characteristics following red cell blood lysis. (A) Gating strategy and histograms showing signals for FITC-labeled Fab-specific anti-bovine IgG antibodies bound to FcγRs of neutrophils, $CD3^{-}CD56^{dim}$ NK cells and monocytes (dark gray-filled histogram). FcγR-negative $CD3^{+}$ T cells were used as negative controls (black line histogram), which exhibited the same background fluorescence in FL-1 as monocytes treated in a separate preparation with bovine $F(ab)_2$-fragments and anti-bovine F(ab)-FITC (data not shown); light gray-filled histograms represent samples without added bovine IgG. 0.2 mg/mL bovine IgG was used. (B) Dose-dependent binding of bovine IgG by FcγRs of human immune cells is shown. (C) The inhibition of binding of bovine IgG to FcγRs of human immune cells following preincubation of cells with anti-CD16/32/64 blocking Abs is shown. Data shown are representative of three experiments performed.

targets (25.8%) but not of $gp140^{-}$ target cells (2.08%, Fig. 2A, right panel). Nonimmune bovine colostrum IgG did not mediate any killing above levels observed for HIV-negative serum or targets not opsonzised (Fig. 2B, left panel). In contrast, bovine IgG purified from two cows vaccinated with gp140 (Fig. 2B, middle and right panels) mediated HIV-1-specific killing of $gp140^{+}$ targets at levels comparable to the positive control. Bovine IgG-mediated killing was specific for HIV-1 as only low background killing of target cells was detected in the absence of gp140.

Monocytes drive IgG-mediated effector function against target cells in the RFADCC assay

The $CSFE^{-}PKH26^{+}$ cells detected in response to either HIV-1-immune serum or anti-HIV-1 bovine colostrum IgG appear to derive from the unstained PBMC population (Fig. 3A, left panels). When $CSFE^{-}PKH26^{+}$ target cells were backgated onto FSC/SSC profiles, events were located within the monocyte region suggesting an association between the killed target cells and

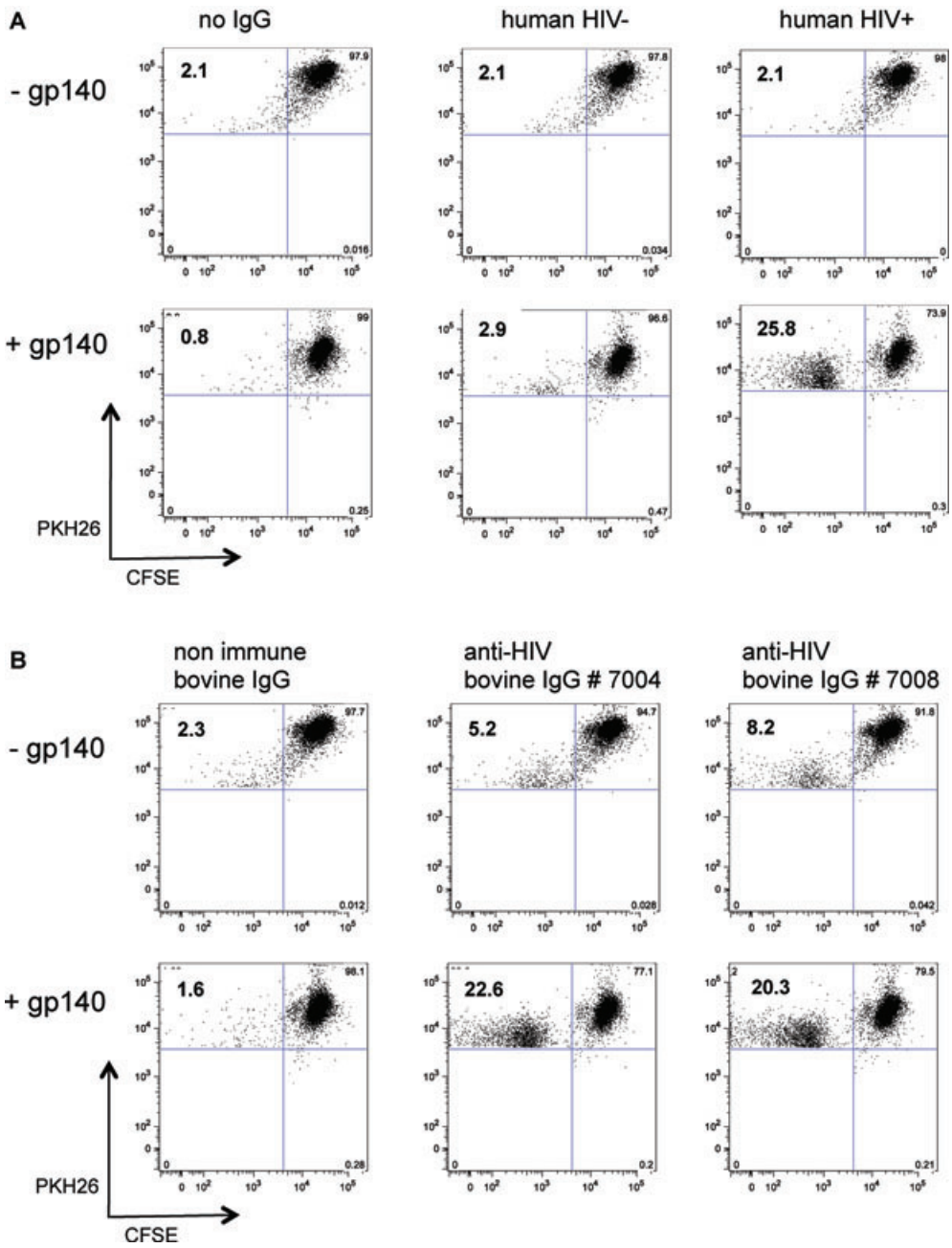

Figure 2. Killing of CEM.NKr-CCR5 target cells in the absence and presence of gp140 mediated by anti-HIV-1 human serum or bovine colostrum IgG. Killing of gp140 pulsed PKH26$^+$CFSE$^+$ CEM.NKr-CCR5 cells by PBMCs in the presence of (A) human HIV-1-immune serum (1:50 dilution) (lower right panel) and (B) bovine colostrum IgG (0.5 mg/mL) (animal 7004 and 7008; lower middle and right panel) was determined by the loss of CFSE after 1h incubation of target and effector cells. The percentage of CFSE$^-$PHK26$^+$ cells is indicated in each top left quadrant. Results are shown for one healthy PBMC donor representative of three donors investigated.

monocytes as effector cells (Fig. 3A, 2nd panels from left). On further analysis, we observed that CFSE$^-$PKH26$^+$ cells stained for the monocyte marker CD14, which is not expressed by CFSE$^+$PKH26$^+$ CEM.NKR-CCR5 cells (Fig. 3A, 3rd panels from the left). A population characterized by high mean fluorescence intensity of PKH26 was observed in CD3$^-$CD14$^+$ cells for gp140$^+$ targets in the presence of HIV-1-immune serum or anti-HIV bovine colostrum IgG (histogram in Fig. 3A, right panels), but not for gp140$^-$ targets (data not shown).

To confirm that monocytes mediate bovine IgG-mediated effector function, we repeated the experiment with purified monocytes at two different Target:Effector ratios, 1:10 (Fig. 3B, top panel) and 10:1 (Fig. 3B, bottom panel). Consistent with the results presented above, no uptake of PKH26 following coculture

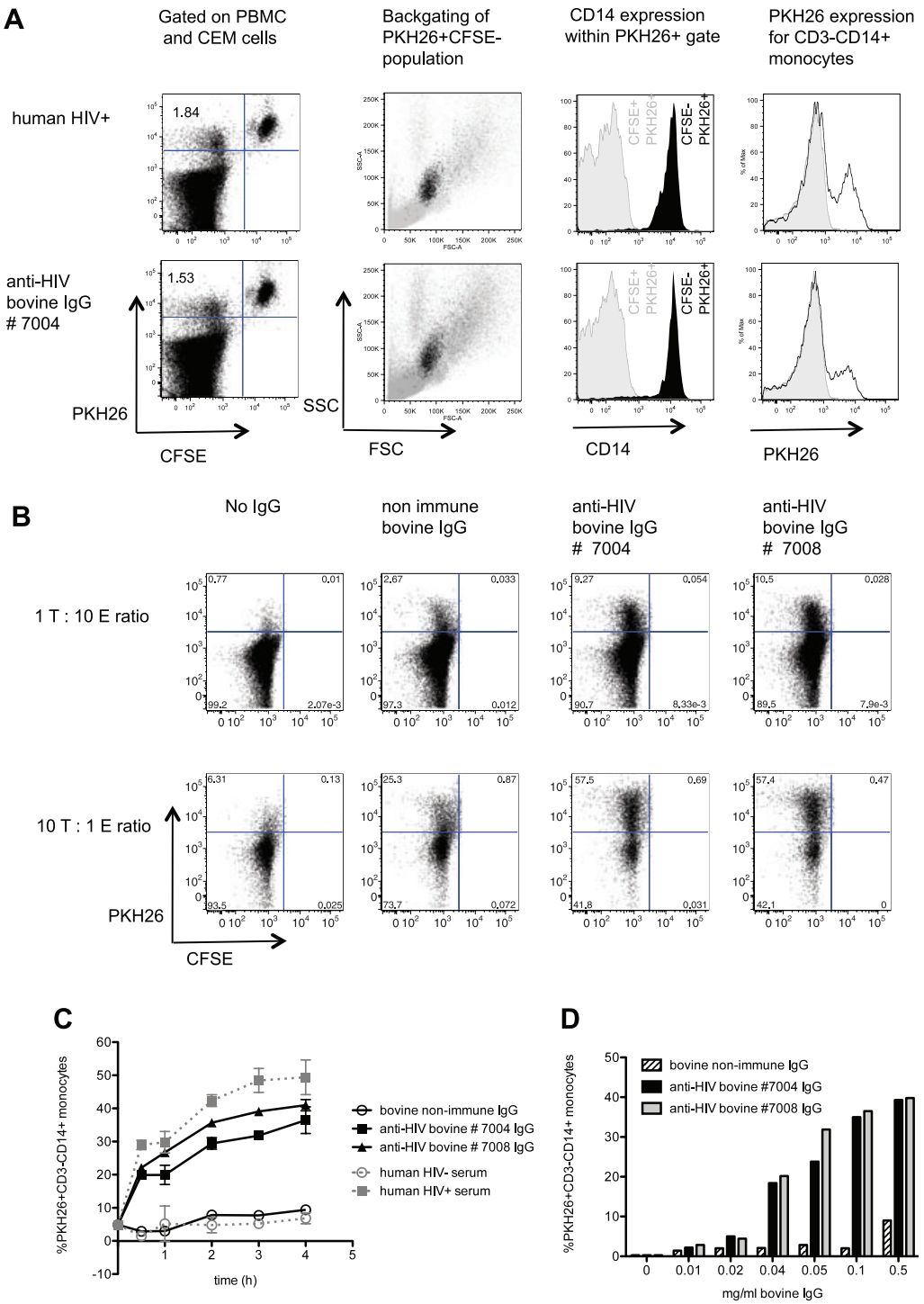

Figure 3. CFSE$^-$PKH26$^+$ cells are monocytes, specifically activated by gp140 pulsed CEM cells opsonized with anti-HIV-1 human and bovine IgGs. (A) Dot plots show PKH26 and CFSE staining in the RFADCC assay using human HIV-1-immune serum and bovine anti-HIV IgG. Backgating of CFSE$^-$PKH26$^+$ cells (left quadrant) onto the forward and side-scatter profiles (FSC, SSC) show the location within the monocyte region (second panels, black dots). Histograms show CD14 expression on CFSE$^-$PKH26$^+$ cells (black-filled histograms) and CFSE$^+$PKH26$^+$ cells (gray-filled histograms). PKH26 fluorescence for CD14$^+$ monocytes in the presence of 1:50 dilution of human HIV-immune serum or 0.50 mg/mL bovine colostrum IgG (open histogram) in comparison to a no IgG control (gray-filled histogram) following 1 h of incubation is shown. (B) Monocyte-mediated ADCC of gp140 pulsed CEM.NKr-CCR5 cells opsonized with bovine colostrum IgG using purified human monocytes in a 1:10 and 10:1 target: effector cell ratio. Results are shown for a concentration of 0.5 mg/mL IgG following 4h incubation. (C) Time course of PKH26 signal in CD3$^-$CD14$^+$ monocytes following incubation of gp140 pulsed CEM.NKr-CCR5 cells in the presence of 1:50 dilution of HIV-1-immune serum and 0.5 mg/mL of bovine anti-HIV-1 colostrum IgG. Results are shown as mean ± SD obtained from PBMCs of three different HIV-negative blood donors. (D) Titration of bovine colostrum IgG in regard to PKH26 signal in CD3$^-$CD14$^+$ monocytes following 4 h incubation. Data are shown for one healthy PBMC donor and are representative of three experiments performed.

with gp140$^+$ target cells was observed in the absence of IgG. For both Target:Effector ratios low background rates of PKH26 uptake from gp140$^+$ targets were observed in the presence of nonimmune bovine colostrum IgG. However in the presence of anti-HIV-1 bovine colostrum IgG-specific effector function against gp140$^+$ targets was at least 2.5-fold higher. Bovine colostrum IgG did not induce PKH26 uptake from gp140$^-$ targets (data not shown). Monocytes did not become PKH26$^+$ at 4°C (data not shown) excluding the possibility that PKH26$^+$ targets are non-specifically adhering to monocytes as effector function but not binding is abolished at 4°C.

Anti-HIV-1 bovine colostrum IgG-mediated effector function is time and dose dependent

To further define the potency of the IgG-mediated effector function of anti-HIV-1 bovine colostrum IgG, we analyzed the kinetics and concentration dependence of the ADCC activity. Within 30 min after gp140$^+$ targets opsonized with bovine anti-HIV-1 colostrum IgG, were mixed with PBMCs, HIV-1-specific PKH26 uptake was detectable and increased over time (Fig. 3C). The kinetics of PKH26 uptake indicating ADCC by monocytes was similar when target cells were opsonized with human HIV-immune serum. The magnitude of PKH26 uptake over time was independent of the healthy PBMC donor used ($n = 3$). Ab-mediated PKH26 uptake was also dose dependent, with detectable ADCC activity increasing from the lowest concentration (0.01 mg/mL IgG) to the highest concentration (0.5 mg/mL IgG) tested (Fig. 3D). The concentration dependence is consistent with that of binding opsonized target cells to monocytes (Fig. 1B above).

Monocyte ADCC requires binding of Fc-fragment to the FcγRs

To confirm the requirement of the Fc region of IgG for the observed effector function, we generated F(ab')$_2$ fragments from anti-HIV-1 and nonimmune bovine colostrum IgG by pepsin digestion. Western blotting confirmed high purity of all F(ab')$_2$ fragments, as shown in Fig. 4A in comparison to undigested IgG. ELISA confirmed potent binding of F(ab')$_2$ fragments to gp140 (Fig. 4B). DCC activity of intact IgG (Fig. 4C, left panels), F(ab')$_2$ fragments (Fig. 4C, middle panels) and Fc-fragments after protein G purification (Fig. 4C, right panels) was assessed by gating on CFSE$^-$PKH26$^+$ cells. No PKH26 uptake was observed in the presence of F(ab')$_2$-fragments or Fc-fragments alone, whereas intact bovine IgG mediated strong effector function. This confirmed that PKH26 uptake is Fc-dependent and not only requires specific binding of antigen and F(ab')$_2$ fragment but also requires binding of the Fc domain to the respective FcγRs. Similarly, CD3$^-$CD14$^+$ monocytes become PKH26$^+$ only in the presence of intact IgG but not in the presence of F(ab')$_2$-fragment or Fc-fragment (Fig. 4C).

ADCC mediated by anti-HIV-1 bovine colostrum IgG mainly utilizes FcγRIIa

To further characterize the mechanism involved in monocyte-ADCC mediated by bovine IgG, we used anti-FcγRIIIa (CD16a), anti-FcγRIIa (CD32a), and anti-FcRI (CD64) antibodies to selectively block the respective FcγR (5 μg/mL; as previously described [26–28] in the following combinations: (i) anti-CD16, anti-CD32, or anti-CD64 alone, (ii) double combinations of either anti-CD16/CD32, anti-CD16/CD64, or anti-CD32/CD64, or (iii) all three anti-CD16/CD32/CD64 antibodies together. Single use of anti-CD16 and anti-CD64 had no or little impact on inhibiting monocyte-mediated ADCC (17.8% and 9.6% inhibition, respectively). In contrast, anti-CD32 alone inhibited PKH26 uptake by 36.2%, which was further increased if used in combination with anti-CD64 (62.0% inhibition) or anti-CD16/CD64 (64.0% inhibition) (Fig. 5). Blocking with anti-CD32 together with anti-CD16 did not boost ADCC inhibition. A similar pattern of monocyte-ADCC inhibition was observed when human HIV-immune serum was used although overall inhibition was much lower compared to bovine IgG.

Discussion

We found here that bovine colostrum IgG with broad cross-clade binding ability to gp140 bind efficiently to human FcγR-bearing innate immune cells and mediate HIV-1-specific killing equivalent to human IgG. FcγR-mediated ADCC has the potential to provide a rapid response against HIV-1-infected cells, which might be particularly important in Ab-mediated approaches for prevention of HIV-1 transmission, such as an antibody-powered topical microbicide. The generation of large amounts of ADCC-mediating bovine colostrum IgG also provides a valuable tool to isolate and study the efficacy of anti-HIV-1 ADCC Abs.

We found that IgG from gp140-immunized but not from non-immunized cows mediated ADCC. We demonstrated that ADCC-mediated killing was specific for anti-HIV-1 bovine colostrum IgG as no killing above background was observed either for nonimmune bovine colostrum IgG or for gp140$^-$ target cells. The magnitude of bovine IgG-mediated killing was very similar to that induced by an HIV-immune serum from an individual with a well-characterized ADCC response. We observed similar kinetics of killing using human and bovine IgG which implies that both types of IgG (i) function in the same mechanism of killing, (ii) are equally functional and (iii) that observed killing is a gp140-specific effect. When F(ab')$_2$ were used, ADCC was reduced to background levels, showing that killing requires interaction of the Fc-domain of bovine IgG with FcγRs which is an established prerequisite for ADCC activity [16, 29]. To our knowledge, this is the first report demonstrating that bovine colostrum IgG can specifically induce cytotoxic effector function of human immune cells.

We initially analyzed IgG-mediated effector function by gating on PKH26$^+$ cells and determining the proportion of CFSE$^-$PKH26$^+$ cells, which were previously defined as killed cells [24]. However,

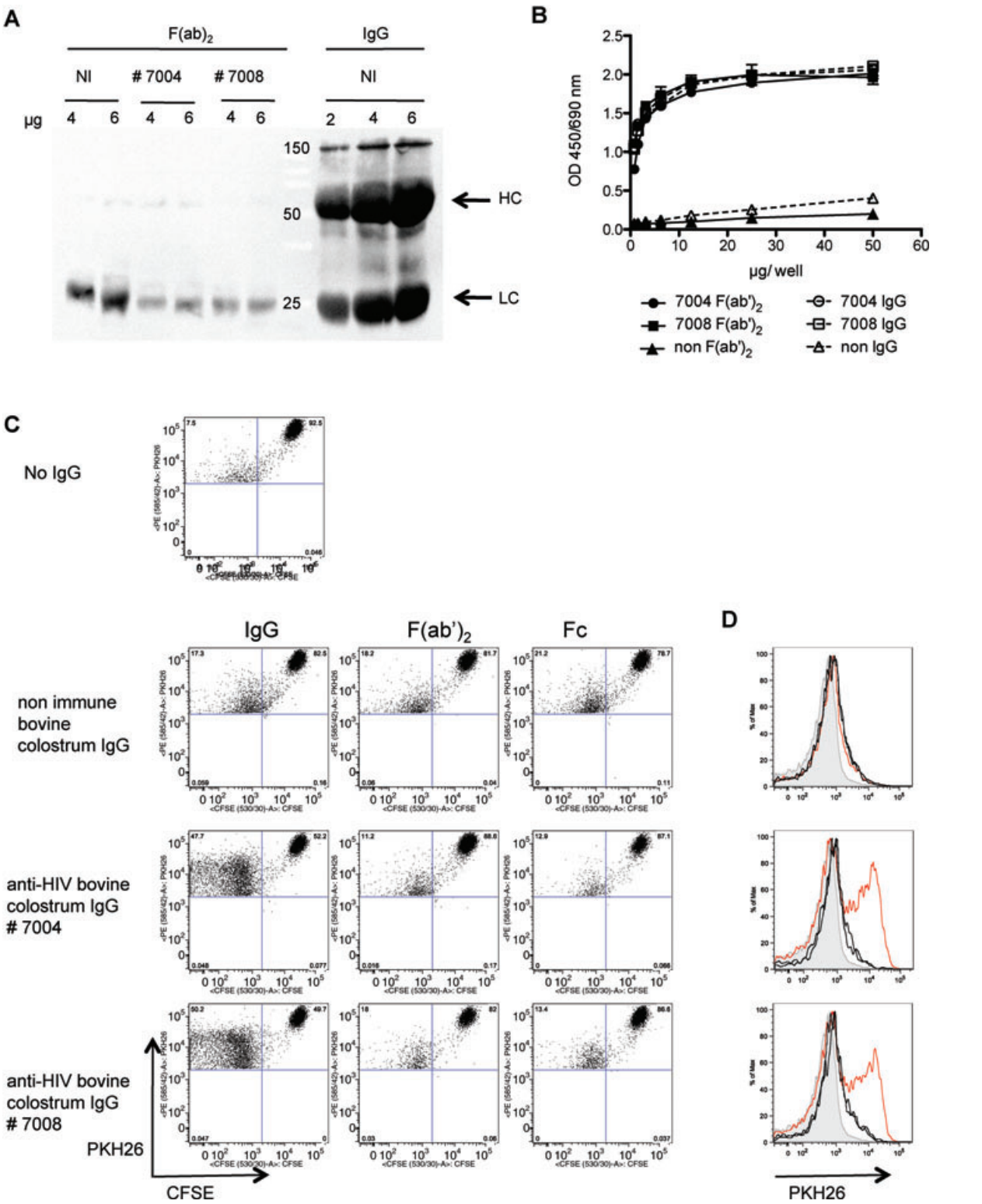

Figure 4. HIV-1 gp140-specific killing of CEM.NKr-CCR5 target cells mediated by bovine IgG is abrogated by removal of the Fc-region. (A) Analysis of F(ab')$_2$ fragments generated from nonimmune (NI) and anti-HIV-1 bovine colostrum IgG (#7004 and #7008) by pepsin digest in comparison with undigested IgG using 15% SDS PAGE under reducing conditions followed by Western blotting; HC: heavy chain, LC: light chain. (B) Confirmation of binding of F(ab')$_2$ fragments to gp140 by direct ELISA. Data are shown as mean of triplicate measurements. (C) RFADCC measuring killing of gp140-coated CEM.NKr-CCR5 cells opsonized with bovine colostrum IgG, F(ab')$_2$ fragments or Fc-fragments. Target cells not opsonized served as a negative control (upper panel). (D) Corresponding histograms showing PKH26 fluorescence within CD14$^+$ monocytes. No IgG: gray-filled histograms, IgG: red line histogram, F(ab')$_2$-fragment and Fc-fragment: black line histogram. Data shown are representative of three experiments performed.

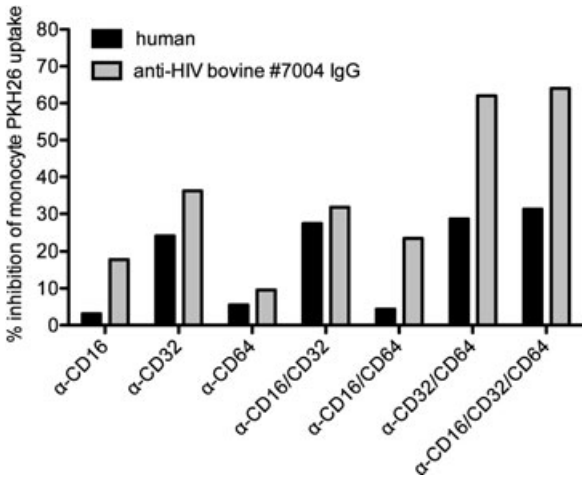

Figure 5. Efficient reduction of bovine IgG induced monocyte-mediated ADCC following surface receptor blocking with anti-FcγRIIa (CD32). Uptake of PKH26 by monocytes following coculture with gp140-coated CEM.NKr-CCR5 cells in the presence of 1:50 dilution of human HIV immune serum or 0.5 mg/mL bovine colostrum IgG after 1.5-h incubation. FcγRs on PBMCs were blocked with 5 μg/mL of each of the indicated specific anti-FcγR monoclonal antibodies for 15 min/37°C, before being used in the RFADCC assay (effector to target cell ratio: 10:1). Inhibition of killing was normalized to percent killing in the absence of blocking antibodies. Data shown are representative of three experiments performed.

the $CFSE^{-}PKH26^{+}$ population appears to be associated with the PBMC population. Further experiments showed that the majority of $CFSE^{-}PKH26^{+}$ events were present in cells, which expressed the monocyte marker CD14 suggesting that monocytes are involved in killing targets. We confirmed the critical involvement of monocytes in this ADCC assay by purifying monocytes [30] and showing their ability to mediate ADCC when anti-HIV-1 bovine colostrum IgG is present. The involvement of monocytes in ADCC has been also shown in the context of malaria [31].

Bovine IgG is able to bind to human FcγR on neutrophils, monocytes, and NK cells in a dose-dependent manner. Strongest binding of bovine IgG was observed for neutrophils and monocytes, which is likely due to the surface expression of the high-affinity FcγRI that is able to bind monomeric IgG [32]. In contrast, efficient binding of IgG to the low-affinity receptors, FcγRIIa and FcγRI, requires immune complex formation, although partial binding of monomeric IgG is possible [33]. This is in agreement with our results that NK cells, the only cell population analyzed, which does not express the high-affinity FcγRI bound bovine IgG less efficiently compared to neutrophils and monocytes. In contrast to previous reports [22], we were able to detect specific binding to FcγRs on NK cells. This may be due to higher IgG concentrations used or a result of possible aggregate formation of IgG at low temperatures allowing it to bind to low-affinity receptors. We did not take measures to remove aggregates in our study. Blocking of FcγRI, FcγRIIa, and FcγRIIIa lead to a reduction in PKH26 uptake, predominately when FcγRIIa was blocked. Although inhibition of killing was incomplete, our results obtained using RFADCC assay indicate that FcγRIIa is the major FcγR involved in monocyte ADCC, in accordance with a previous study [34]. These data might warrant future studies into CD32 polymorphisms on killing activity.

Although data suggests that Abs with ADCC-specific epitopes in humans are associated with nonprogression [25], it is unclear whether ADCC Abs can provide sterile protection. To study and define ADCC-specific epitopes and how these differ from those inducing neutralizing activity is technically demanding, as ADCC-specific but nonneutralizing IgG are scarce in human plasma. HIV-1-hyperimmune bovine colostrum, however, contains large amounts of IgG providing an excellent opportunity to purify epitope-specific ADCC Abs and test them in passive transfer experiments. Bovine colostrum IgG will be a valuable tool to clarify the role of ADCC in HIV-1 protection in the absence of neutralization.

In conclusion, anti-HIV bovine colostrum IgG mediate robust HIV-1-specific killing and stimulate functional response in human cells in vitro. Such FcR-mediated cell function has the potential to provide rapid and potent responses against HIV-1 and may be of particular interest for Ab-mediated approaches for HIV prevention and to study efficacy of anti-HIV-1 ADCC Abs in vivo.

Materials and methods

Generation of anti-HIV-1 bovine colostrum IgG by vaccination of cows

Anti-HIV-1 bovine colostrum IgG was produced by vaccination of cows with 100 μg oligomeric gp140 of clade B (AD8) (#7004) or with a mixture of clade A (UG8), clade B (AD8), and clade C (MW) (#7008) once before and twice after conception. Protein G-purified polyclonal IgG from HIV-1 hyperimmune but not from nonimmune bovine colostrum demonstrate efficient binding to gp140 Env of clade A, B, and C (endpoint concentrations between 1.56 μg/μL and 6.26 μg/μL) and potent cross-clade neutralizing activity has been shown for all tested viruses ($n = 10$, IC_{50} of 1 mg/mL) [23].

Preparation of purified bovine colostrum IgG and F(ab')2 fragments

Colostrum IgG was purified as previously described [35]. Briefly, colostrum was defatted and pasteurized by centrifugation at 10,000 × *g* for 30 min at 4°C and incubation at 63°C for 30 min. Casein was depleted by adjusting the pH to 4.6 with an equal volume of 0.2 M sodium acetate solution (pH 4.0) followed by cooling and centrifugation at 10,000 × *g* for 30 min and adjustment to pH 6.6. Colostral whey was dialyzed against PBS using a 30 kD cut-off ultrafiltration membrane (Amicon Ultra 15 mL, Millipore). Bovine IgG was purified by protein-G sepharose chromatography (GE Healthcare) and then dialyzed against PBS.

For preparation of $F(ab')_2$ fragments, pepsin (10 mg/mL in 0.2 M sodium acetate buffer pH 4.0) was added to colostral whey in a ratio of 1:20 followed by incubation at 37°C for 20 h. Digestion was stopped by placing on ice and pH adjustment to 7.4 with 1 M Tris-HCl followed by overnight dialysis against 20 mM sodium phosphate and 150 mM NaCl at pH 6.0 at 4°C. To remove excess pepsin, sample was applied to a 10-mL Q-Sepharose column and the flow through that contained $F(ab')_2$ fragments, Fc-fragments, and undigested IgG was collected and adjusted to pH 7.4 using 1 M Na_2HPO_4. $F(ab')_2$ fragments were separated from intact Ig and Fc fragments by protein-G sepharose chromatography by collecting the flow through. Column-bound IgG and Fc-fragments were eluted and used as a control.

Purified IgG and $F(ab')_2$ fragments were filter sterilized and concentrations were measured by absorbance at 280 nm using a molar absorption coefficient of 1.35 and 1.18, respectively.

SDS page and western blotting

Bovine IgG and $F(ab')_2$ fragments were resolved by 15% reducing SDS-PAGE, transferred to PVDF membrane and blocked with 5% casein in PBS overnight at 4°C. IgG was detected using HRP-conjugated rabbit anti-bovine IgG antibody (1:1000 dilution, Sigma) and Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) according to manufacturer's instructions. Bands were visualized using Kodak 4000 MM Image Station and IM software (Carestream Health).

Envelope-binding ELISA

Purified gp140 in 100 μL/well coating buffer (pH 9.8; 2mM Tris, 10mM NaCl) was coated onto 96-well polyvinyl-flat bottom plates (Pathtech, Australia) at 100 ng/well overnight at 4°C. Wells were washed sequentially in PBST (PBS/0.1% Tween-20) and PBS and then blocked with 5% Casein in PBS for 2 h. Wells were washed as described above, followed by the addition of 2-fold dilutions of purified IgG and $F(ab')_2$ fragments in 5% casein. After a 4 h incubation, wells were washed and horseradish-peroxidase-conjugated (HRP) rabbit anti-bovine IgG antibody (Sigma) was added and incubated for 1 h. Color reactions were developed using 3,3′ -5,5′ -Tetramethylbenzidine (TMB) and absorbance was measured at 450 nm against a reference of 690 nm.

Whole-blood flow cytometric assay for bovine IgG binding to human Fc receptors

Blood was collected (with approval of the University of Melbourne and Alfred Hospital Human Research and Ethics committees and informed consent of all participating subjects) from healthy blood donors into EDTA anticoagulation tubes and washed once with 1× PBS. One hundred microliters of washed blood was incubated with 5–40 μg of purified bovine IgG for 30 min at room temperature. After washing excess/unbound bovine IgG with 1× PBS, bound IgG was detected by incubating with anti-bovine $F(ab')_2$ (goat) FITC antibody (Rockland) at manufacturer's recommended concentration for 30 min at 37°C. Cells were washed once with 1× PBS. Cells were then labeled with phenotyping antibodies for 20 min at room temperature followed by red blood cell lysis using BD lyse solution (BD Biosciences 349202). Cells were washed again with 1× PBS, fixed in 1% formaldehyde, and analyzed using a FACSCalibur and FlowJo analysis software version 9.4.9. Antibodies used: of anti-human CD3-PerCP-Cy5.5 (BD Biosciences, 340949), anti-human CD56-allophycocyanin (Beckman Coulter IM2474), and anti-human CD16-PE (BD Biosciences, 347617).

Cells

CEM.NKr-CCR5 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and maintained according to provider's protocol. PBMCs were isolated from heparin blood from healthy donors and monocytes from buffy packs provided by the Melbourne Red Cross Blood Bank. PBMCs were prepared by Ficoll density gradient centrifugation. Monocytes were purified from PBMCs by counter-current elutriation using a Beckman Coulter J-6M/E centrifuge equipped with a JE-5.0 rotor, at 2200 rpm, 12°C. Fractions were analyzed during the run by flow cytometry, using light scatter characteristics to estimate monocyte content, and fractions containing greater than 88% monocytes were collected and pooled. Typical monocyte purity was above 90%.

RFADCC assay

The RFADCC assay was performed as previously described [24], and 1×10^6 CEM.NKr-CCR5 cells were coated with 3 μg of purified gp140 AD8 (production of gp140 described elsewhere [36]) in 50 μL of RF10 media for 1 h at room temperature. Uncoated CEM.NKr-CCR5 cells were treated identically but without adding gp140 AD8 protein. Coated and uncoated CEM.NKr-CCR5 cells were first labeled with PKH26 (Sigma) and then with CFSE (Sigma) according to manufacturer's instructions, and 2×10^4 $PKH26^+CFSE^+$-labeled CEM.NKr-CCR5 target cells were opsonized with either human IgG (serum from HIV-1-infected and HIV-uninfected individuals), with bovine IgG or bovine $F(ab')_2$-fragments for 30 min at 37°C. 2×10^5 PBMCs or purified monocytes were always added to target cells at a target-to-effector cell ratio of 1:10 unless stated otherwise. Cells were incubated for 1–4 h at 37°C as indicated, then stained for CD3 (anti-human CD3-PerCp, BD Biosciences, clone SK7), and CD14 (anti-human CD14-allophycocyanin-H7, BD Biosciences, clone MøP9). After 30-min incubation at room temperature, cells were washed once with 1× PBS, fixed in 1% formaldehyde, and analyzed using a FACS-Canto II cytometer and FlowJo analysis software (Version 9.4.9.).

Blocking of FcγR

For blocking experiments, the RFADCC assay was performed as described above with the exception that PBMCs were incubated for 30 min at 37°C with the following FcγR blocking antibodies at 5 μg/mL: anti-human CD32 clone 6C4, (eBioscience); anti-human CD16 clone 3G8 and anti-human CD64 clone 10.1 (both Biolegend) before CEM.NKr-CCR5 cells and PBMCs were mixed together. Concentrations of blocking antibodies were used according to manufacturer's specifications.

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Conflict of interest: GR is Chief Scientific Officer and a shareholder in Immuron Limited. DFP is shareholder of Immuron Limited. GR, DFP, MK, and RJC are named inventors on a pending patent. All other authors declare no financial or commercial conflict of interest.

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Abbreviations: **ADCC**: antibody-dependent cellular cytotoxicity · **NAb**: neutralizing antibody

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