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Research paper

Granulocyte contamination dramatically inhibits spot formation in AIDS virus-specific ELISpot assays: Analysis and strategies to ameliorate

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Abstract

The interferon-γ (IFNγ) ELISpot assay has become the most critical tool for HIV vaccine evaluation. External factors affecting ELISpot results must be minimized for the data to be reliably used in vaccine research and development processes. In pre-clinical pigtail macaque studies analyzing HIV/SIV vaccine studies, we detected a strong correlation between levels of granulocytes contaminating PBMC preparations and reduction in the quality and quantity of spots in the IFNγ ELISpot assay. Acute SHIV infection of macaques worsened granulocyte contamination of PBMC fractions and made the assay much less reliable in detecting SIV-specific T cell immunity compared to intracellular cytokine staining (ICS). This problem could be ameliorated by using an $F(ab)_2$ form of the MD-1 IFNγ capture antibody, presumably reflecting that activation of granulocytes in the well by the Fc portion of the standard capture antibody disrupts spot formation. Improving the standard ELISpot assay by using an $F(ab)_2$ capture antibody should make it more reliable for use in critical vaccine development studies.
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1. Introduction

New vaccines for many global pathogens such as HIV are likely to require the induction of cellular immune responses. Reliable measures of cellular immune responses in outbred subjects (such as macaques and humans) are essential for HIV vaccine development. The cytokine ELISpot assay for T cell immune responses (Czerkinsky et al., 1988) was adapted from the B cell antibody assay (Czerkinsky et al., 1983) and has since become widely accepted in

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laboratories evaluating functional responses to vaccines in both pre-clinical and clinical trials. The ELISpot assay is a modification of the indirect or capture ELISA and enumerates cytokine expressing mononuclear cells rather than overall cytokine concentration in the well. A plate-bound anti-cytokine mAb captures cytokines released from activated cells and cytokine production in the form of a "spot" is typically detected with a labelled second anti-cytokine mono- or polyclonal antibody followed by one of several different spot visualisation systems.

The ELISpot assay has been reported to measure combined CD4 and CD8 T cell precursor frequency with a sensitivity of 50 antigen-specific T cells per million cells (Russell et al., 2003) that is comparable to or better than other immunological assays such as cytokine ELISA assays (Tanguay and Killion, 1994) and the ICS assay (Karlsson et al., 2003). Unlike the T cell proliferation assay and the labour intensive ^{51}Cr-release CTL assay it does not require in vitro expansion of antigen-specific cells and so provides a rapid and undistorted direct measure of the immune response ex vivo. The ELISpot assay's typical incubation time of 20–24 h also accommodates antigens in the form of proteins (Schmittel et al., 2001) and whole inactivated virus (Dale et al., 2004b; Gloster et al., 2004) which require capture and processing prior to presentation on MHC II complexes, in addition to peptide antigens that can bind directly to MHC I complexes (Kumar et al., 2001). Practical advantages include the relative simplicity of the procedure itself and it's amenability for high sample throughput. Enumeration of the number of spot forming units can be performed by microscopy although automated ELISpot readers are desirable when sample or spot numbers are high and for consistency of well to well measurement. The ELISpot assay has become the cellular immune assay generally employed to determine primary immunogenicity endpoints in many clinical trials and critical vaccine development decisions rely on precision with this assay (Russell et al., 2003).

Vaccine trials necessarily involve direct comparisons of responses between individuals or groups of participants. It is critical therefore to ensure that little or no extraneous factors are introduced into the assay that can adversely affect this comparison. Peripheral blood mononuclear cells (PBMC) prepared by Ficoll gradient density centrifugation of whole blood, which are the starting cultures for these assays represents a potential source of variation. We recently observed that preparations of macaque PBMC occasionally contain low-density granulocytes that co-purify with lymphocytes and monocytes at the Ficoll/plasma interface. During acute SHIV infection of macaques this undesired population can increase. Contaminating granulocytes strongly correlate with poor spot forming ability of both vaccine-specific and superantigen-induced T cell responses. We show that this affect can be dramatically ameliorated by using a truncated $F(ab)_2$ IFNγ-specific capture antibody coated onto the plate, likely reflecting activation of granulocytes by the Fc portion of the standard antibody.

2. Materials and methods

2.1. Monkeys

PBMC samples were sourced from 56 juvenile pigtail macaques (*Macaca nemestrina*) negative for HIV-1/SIV and simian retrovirus (SRV) infection and housed in physical containment level 3 conditions. Anaesthesia was induced prior to all procedures using ketamine (10 mg/kg) administered intramuscularly. Experiments were performed according to guidelines on the care and use of laboratory animals issued by the National Institutes of Health and were granted approval by the Animal Experimentation and Ethics committees from the University of Melbourne and CSIRO Livestock Industries.

2.2. Vaccine trials

DNA and recombinant fowl pox virus (rFPV) prime/boost vaccine regimens induce high level T cell responses as previously shown (Kent et al., 1998). PBMC samples were derived from two vaccine trials utilizing DNA and rFPV vaccines encoding SHIV antigens (Dale et al., 2004a). Macaques received 1–3 DNA vaccinations and a single rFPV booster or control DNA and rFPV vaccines. Ten weeks after the final vaccine, all animals were challenged with pathogenic $SHIV_{mn229}$ or $SHIV_{SF1623}$ (Harouse et al., 2001; Dale et al., 2002).

2.3. PBMC preparation

PBMC were prepared from heparin-anticoagulated blood by density gradient centrifugation (1000×*g*, 25 min, 24 °C) with Ficoll-Paque PLUS (Amersham Biosciences, UK) diluted to 95% with sterile, deionized water to improve separation of pigtail macaque PBMC. Cells were washed twice (500×*g*) with 15 ml of RPMI at 10 °C, resuspended in 0.5–1 ml of RPMI and stored on ice/1–2 h prior to assay. PBMC concentrations were measured with a Coulter A^C.T diff Analyzer (Beckman Coulter, Fullerton, CA) and cell concentrations adjusted with RPMI containing 5% fetal calf serum (RF5).

2.4. IFNγ ELISpot assay

ELISpot assays were performed with freshly isolated PBMC using the monkey IFNγ ELISPOT assay system (U-Cytech, Utrecht, The Netherlands) in 96-well flat-bottomed, transparent microtitre plates (Greiner Bio-One, The Netherlands). Wells were coated with 50 μl of anti-IFNγ mAb MD-1 (U-Cytech) at 10 μg/ml in PBS and incubated overnight at 4 °C. Alternatively, wells were coated with an $F(ab)_2$ derived MD-1 mAb (U-Cytech) at the same concentration. Plates were washed 5 times with PBST (PBS containing 0.05% Tween 20) to remove loosely bound capture antibody and blocked with PBS containing 1% BSA for 1 h at 37 °C. The blocking agent was removed immediately prior to the addition of cells (no washes). PBMC (5×10^5 in 300 μl) were cultured separately in 48-well plates (Corning, NY) for 18 h at 37 °C and 5% CO_2 with a single pool of 125 SIV Gag peptides at 1 μg/ml/peptide (obtained through the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH), aldrithiol-2 inactivated SIV_{mac239} particles and control microvescicles at 5 μg/ml (kindly provided by Dr Jeff Lifson, National Cancer Institute) or the superantigen SEB (Staphylococcal enterotoxin B, Sigma, St. Louis, MO) at 10 ng/ml. Cells were washed twice with 1 ml RPMI with 5% FCS (500×*g*) while simultaneously reducing the volume of suspension to 200 μl prior to transfer into ELISpot plates. This pre-stimulation step improves the signal/noise ratio of spots by reducing background resulting in improved reliability, particularly for enumerating wells with high numbers of spot forming units such as mitogen-activated cells (not shown). Antigen was added to the washed, pre-stimulated PBMC and 2.5×10^5 cells in 100 μl were transferred to the ELISpot plates in duplicate wells for a further 5 h incubation step at 37 °C and 5% CO_2, then stored overnight at 4 °C. PBMC were discarded and remaining cells lysed by incubating with 200 μl ice-cold deionized water for 10 min. Cellular debris was removed by washing 10 times with PBST. Captured IFNγ was detected by 1 h incubation at 37 °C with 100 μl of rabbit polyclonal, biotinylated anti-IFNγ (pAb, U-Cytech) at 0.5 μg/ml in 1% BSA in PBS and then washed 5 times with PBST. 50 μl of anti-biotin IgG gold conjugate (GABA, U-Cytech) at 0.2 μg/ml in 1% BSA in PBS was added to each well for 1 h at 37 °C and washed 5 times with PBST. Sites of cytokine deposition were revealed with the formation of silver salt precipitates on the gold label using 30 μl of activator mix (U-Cytech) per well for 30 min incubation at room temperature in the dark. Plates were washed several times with tap water and air-dried prior to counting. Spots were counted using an ELISpot reader system (model ELR02, AID, Germany).

2.5. ICS

To compare ELISpot results on PBMC with a method detecting antigen-specific responses on unfractionated blood, intracellular IFNγ secretion was assessed by flow cytometry as previously described (Maecker et al., 2001; Dale et al., 2002). In short, 200 μl whole blood was incubated with peptides as described above at 1 μg/ml along with co-stimulatory antibodies anti-CD28 (clone L293) and anti-CD49d (clone L25.3) (BD Biosciences PharMingen, San Diego, CA) for 2 h at 37 °C and 5% CO_2. Antigen processing was then blocked by the addition of 10 μg/ml Brefeldin A (Sigma), and incubated for a further 4–5 h. The cells were then stained with anti-CD3-PE (clone SP34) and anti-CD8-PerCP (clone SK1) (BD Biosciences) for 30 min at 4 °C. Erythrocytes were then lysed using FACS lysing solution (BD Biosciences) washed with PBS and the remaining cells permeabilised with FACS permeabilising solution (BD Biosciences). Cells were then incubated with anti-IFNγ-allophycocyanin (APC; clone B27, BD Biosciences) and formaldehyde-fixed

before acquisition (BD FACScalibur). Antigen-specific CD8+ T cell responses were assessed as the percentage of CD3+CD8+ cells expressing IFNγ above DMSO control-stimulated cultures.

2.6. Statistical analysis

To determine whether granulocyte contamination of PBMC suspensions contributed to lower spot counts in the ELISpot assay, 782 coordinates cross-referencing granulocyte percentage with the number of spot forming units/million PBMC in SEB-activated samples were plotted and analyzed by simple and random (that accounts for re-sampling from the same animal) regression correlation models. Analysis of the putative anomaly between pre- and post-challenge immune responses in the ELISpot assay was assessed by a comparative analysis of the respective regression slopes and sample group variances.

3. Results

3.1. Granulocytes in PBMC preparation impact on spot quality

Subpopulations within freshly isolated PBMC preparations from pigtailed macaques were enumerated using a Coulter counter with differential leukocyte analysis. This unit allows macaque lymphocytes and monocytes/granulocytes to be differentially counted on the basis of volume after treatment with a lytic reagent that alters the properties of these cells. Macaque lymphocytes form a single, low volume, peak on differential plots, while monocytes and granulocytes form a single combined peak to the right of the lymphocyte peak (Fig. 1). Since the proportion of monocytes in macaque whole blood is generally low (<10% on manual differential counts, data not shown) the

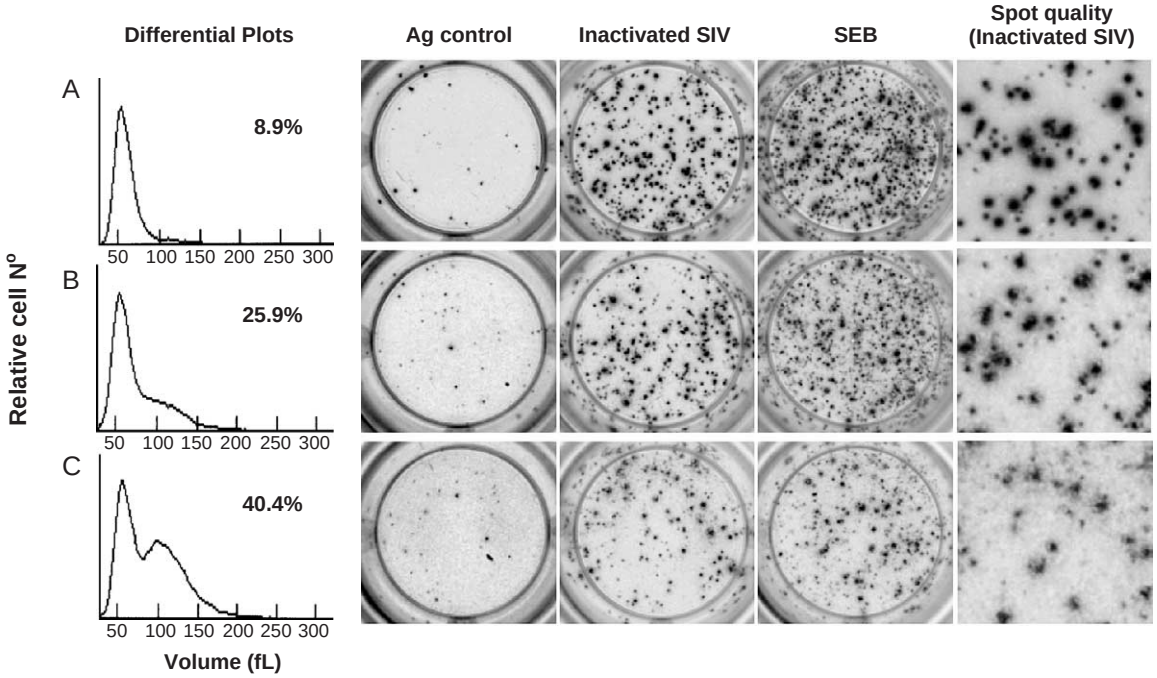

Fig. 1. Granulocyte contamination reduces spot quality. Freshly prepared PBMC samples from 3 macaques containing (A) low, (B) moderate, and (C) high levels of contaminating granulocytes were restimulated with inactivated SIV_{mac239} particles, control antigen or the superantigen, SEB and spot quality compared. Differential plots from a Coulter counter display lymphocyte (0–90 fL) and monocyte/granulocyte (>90 fL) peaks (monocytes generally comprise ~5% of the second peak). Inactivated SIV=supernatant fluid from SUPT1-CCR5 CL30 cell line expressing SIV_{mac239} particles, inactivated with Aldrithiol-2. Antigen control=supernatant fluid from non-transduced SUTP1-CCR5 CL30 cells. SEB=staphylococcal enterotoxin B.

variation in the second peak above this baseline level is due to contaminating granulocytes in the PBMC preparations.

We initially observed sporadic poor spot quality formation in ELISpot assays from some macaques with high granulocyte contamination (Fig. 1). Investigation of spot forming irregularities in a number of macaque samples revealed a relationship with the proportion of cells in this second peak. When the second peak was below 20% of the total PBMC count spot formation was always intense and with a regular edge. Above 20%, the intensity and size of the spots were diminished and they became less regular. This effect was evidenced in both antigen-stimulated wells and control superantigen-stimulated wells from macaques in SHIV vaccine studies (Fig. 1). When levels of granulocytes reached 40%, spot production was dramatically affected.

3.2. Granulocyte contamination reduces spot numbers

We then thoroughly investigated the relationship between granulocyte contamination and number of spot forming units in the ELISpot assay following superantigen stimulation in 782 samples from 56 pigtailed macaques in 2 large SHIV vaccine studies (Fig. 2). Granulocyte contamination was strongly associated with reduced spot counts, with a highly significant association (r=0.45, p<0.001) towards lower spot numbers with increasing granulocytes in this large sample (Fig. 2A). Analyses using random effect regression models that allow for repeat analysis of the samples from the same animal at different timepoints also yielded a highly significant correlation (p<0.001). When granulocyte contamination was bracketed into 10% increments, spot numbers were not affected until the non-lymphocyte cell number exceeded 20% and decreased steadily thereafter with increasing granulocyte contamination of PBMC (Fig. 2B). Although this downward trend in spot counts may, in part, be due to a reduction in the number of T cells in the granulocyte contaminated PBMC preparations, incomplete spot formation induced by granulocytes impacted on spot numbers with reduced spot counts from the ELISpot reader (Fig. 1). Poorly resolving spots were generally not identified with user-defined thresholds for spot intensity and size.

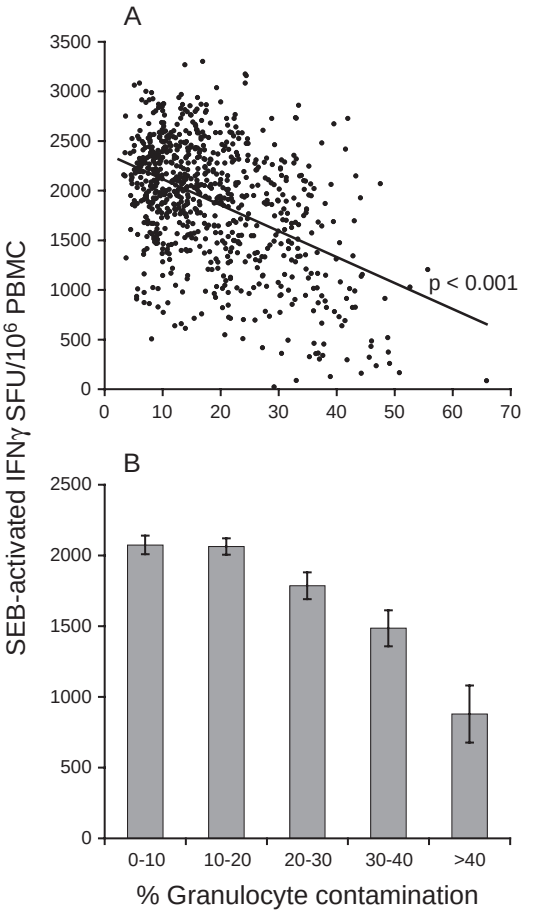

Fig. 2. Effect of granulocyte contamination of PBMC preparations on ELISpot assay performance. 782 SEB-stimulated PBMC preparations collected over multiple timepoints from 56 macaques, studied in SHIV vaccine experiments, were assayed by ELISpot and spot forming units (SFU)/10^6 PBMC compared against the level of granulocyte contamination. (A) Correlation of spot numbers with % granulocytes in individual PBMC preparations, trendline slope, r=0.45 (p<0.001). (B) Comparison of spot numbers in PBMC samples with increasing granulocyte contamination (incremental groups of 10%). Error bars represent 95% confidence intervals.

3.3. Viral challenge increases granulocyte contamination of PBMC suspensions

One of the two vaccine studies involved challenge of macaques with a highly pathogenic $SHIV_{mn229}$ (Dale et al., 2002). Prior to challenge, PBMC were mostly of high quality. We noticed that SHIV challenge appeared to have a negative impact on the

quality of PBMC preparations. We therefore analyzed 467 superantigen-stimulated samples from 36 macaques before and after challenge for a correlation between the number of spot forming units and granulocyte contamination. There were increased numbers of contaminating granulocytes in macaque PBMC samples following SHIV challenge that adversely affected the performance of the ELISpot assay (Fig. 3). A comparison of regression slopes for pre- and post-challenge PBMC samples demonstrated this effect. The difference between the pre- and post-challenge regression slopes was −13.8 spots/% granulocyte contamination (95% confidence intervals −24.8, −2.8, p=0.014).

We then analyzed whether granulocyte contamination impacted not only on superantigen-stimulated PBMC but also on antigen-stimulated samples. For this analysis we compared the efficiency of detecting SIV Gag-specific cell responses by ELISpot assay on PBMC with an ICS assay on whole blood. The impact of viral challenge on the ability of the ELISpot assay to measure SIV-specific cellular immunity was evident when compared directly with flow cytometric analysis of intracellular IFNγ staining (Fig. 4). Although these two assays measure IFNγ expression in different ways, the dynamics of the immune response in SHIV vaccinated macaques measured in each assay were similar after DNA prime and FPV boost vaccination prior to challenge (week 11, when granulocyte contamination of PBMC were lower, mean 11.9%, 99% CI 10.5–13.3) but diverged dramatically by 3 weeks after pathogenic SHIV challenge (week 21, when granulocyte contamination was significantly higher, mean 19.3%,

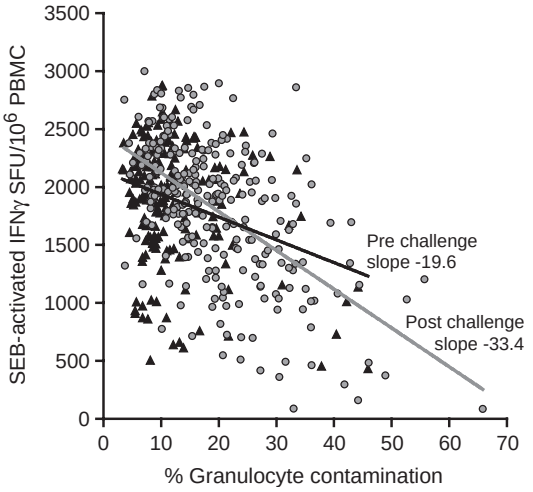

Fig. 3. Impact of viral challenge on ELISpot assay performance. 36 macaques vaccinated with SHIV-encoding DNA and rFPV vaccines or control vaccines were stimulated with the superantigen SEB at multiple timepoints pre- (▲) and post- (●) $SHIV_{mn229}$ challenge and assayed by ELISpot. The numbers of spot forming units (SFU) were plotted relative to percent granulocyte contamination for 467 PBMC preparations. Regression slope analysis of pre-challenge responses was compared with responses post-challenge for statistical differences.

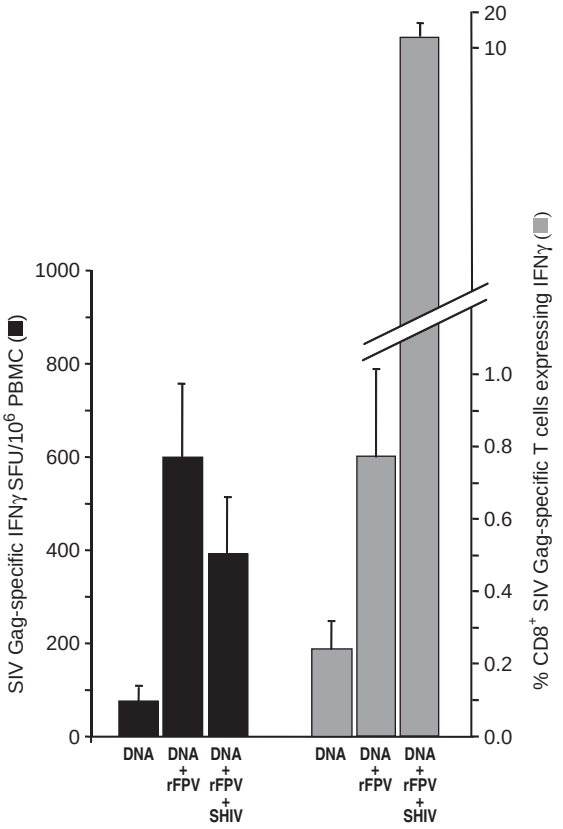

Fig. 4. Relative dynamics of ELISpot and Flow cytometry IFNγ assays. SIV Gag-specific IFNγ expression was analyzed by IFNγ ELISpot and intracellular IFNγ flow cytometry in a group of six macaques receiving SHIV DNA (weeks 0 and 4) and rFPV vaccines (week 8) and later challenged with $SHIV_{mn229}$ (week 18). Group means and standard errors were compared at three timepoints, pre-rFPV boost (week 8), 3 weeks post FPV boost (week 11) and 3 weeks after SHIV challenge (week 21). Responses in ELISpot and ICS assays were normalised to week 11.

99% CI 17.7 and 20.9, $p<0.01$). Gag-specific IFNγ-expressing CD8 T cells were substantially elevated (16.3 fold) in vaccinated macaques after challenge by ICS on whole blood compared with pre-challenge responses, while the ELISpot assay recorded a fall (0.35 fold) in the magnitude of the cellular response, contrary to the expectation that virus challenge would expand pre-primed T cell immunity as demonstrated in previous studies (Amara et al., 2001).

3.4. Minimizing granulocyte interference in ELISpot assays

Two strategies were studied to neutralize or minimize the impact of excessive granulocyte levels on spot formation in stimulated PBMC cultures (Fig. 5). An $F(ab)_2$-modified version of the IFNγ capture antibody, MD-1, was used to investigate whether granulocyte-mediated disruption of spots was due to activation of granulocytes through the Fc receptor

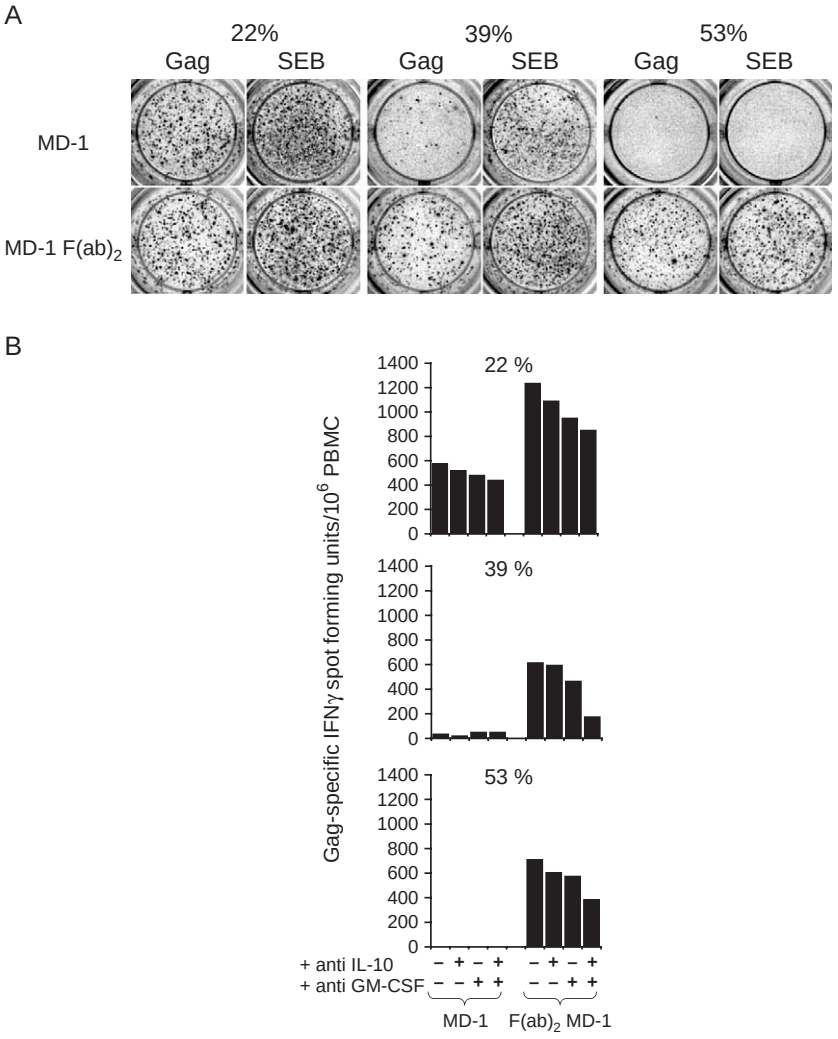

Fig. 5. Improvement in the ELISpot assay associated with a modified capture antibody. (A) PBMC samples containing moderate (22%), high (39%) and v. high (53%) levels of contaminating granulocytes were assayed by ELISpot using the MD-1 capture mAb (top row) and an $F(ab)_2$ version of this antibody (bottom row). SIV Gag-specific and SEB-stimulated responses are displayed for each. (B) Addition of anti-IL-10 or anti-GM-CSF antibodies to cultures did not further improve the detection of IFNγ spot forming units by ELISpot.

(Fig. 5A). Three PBMC cultures containing borderline (22%), high (39%) and very high (53%) levels of contaminating granulocytes were restimulated with SIV Gag peptides or activated with the superantigen SEB in comparative ELISpot assays that used MD-1 and an $F(ab)_2$-modified version of the capture antibody. Little difference was observed in cultures containing 22% granulocytes, although improved definition of spots was observed in the SEB-activated culture. At elevated concentrations of granulocytes within PBMC preparations, clear improvements in spot formation were evident using the $F(ab)_2$ capture antibody that resulted in substantially increased spot numbers. Cultures containing 39% granulocytes produced fewer spots of lower quality when the standard capture antibody was used. While both antigen-specific and SEB activated cells were affected, the impact on antigen-specific restimulation was more severe. At very high levels of granulocyte contamination, spot formation was abrogated in both antigen-specific and mitogen activated cultures. The use of $F(ab)_2$ as a capture antibody dramatically restored spot formation in these wells.

A second strategy to improve the performance of IFNγ ELISpot assay in the presence of a high number of granulocytes was to prevent granulocyte activation by pro-inflammatory cytokines such as IL-10 and GM-CSF. Monoclonal antibodies with high neutralizing activity towards pigtail macaque IL-10 and GM-CSF (U-CyTech) were therefore added to cultures to block these inhibiting factors and ELISpot quality assessed (Fig. 5B). No improvement in spot formation was observed following anti-IL-10 or anti-GM-CSF antibody treatment, either alone or combined. Combining the $F(ab)_2$ antibody with anti-cytokine treatments (anti-IL-10, anti-GM-CSF or anti-IL-10+GM-CSF) also did not have an additive effect.

4. Discussion

This study reports a highly significant correlation between levels of non-lymphocyte cell populations in PBMC and reductions in spot formation detected in IFNγ ELISpot assays conducted during large scale SHIV vaccine studies in pigtail macaques. Contaminating granulocyte populations were increased during acute SHIV infection of macaques and this dramatically inhibited the ability of the ELISpot assay to detect SIV-specific T cell immunity. Remarkably, the inhibiting effect of granulocyte contamination could be ameliorated by use of an $F(ab)_2$ IFNγ capture antibody, presumably reflecting that activation of granulocytes in the well by the Fc portion of the standard capture antibody facilitates the inhibition of spot formation. Improving the standard ELISpot assay by using an $F(ab)_2$ IFNγ capture antibody should make it a more reliable assay to guide vaccine evaluation.

The method to detect granulocyte contamination we used was a Coulter counter that reliably discriminated between lymphocyte and non-lymphocyte PBMC fractions based on cell volume after chemical treatment. Since we determined that monocytes were a constant and small fraction within the non-lymphocyte fraction, the increase in non-lymphocyte fractions within PBMC fractions reflected granulocyte contamination, supported by manual differential counting (data not shown). Future macaque and human studies of this effect could utilize improved Coulter counters able to reliably discriminate between monocytes and granulocytes.

Granulocyte contamination had a dramatic effect not only on superantigen-stimulated cells, but on SIV-specific T cells as well. Although we could reasonably assume a reduced SIV-specific result based on reduced superantigen responses, proving this effect required comparison with an assay not affected by granulocytes, such as the ICS assay, which uses whole blood. A striking and expected increase in SIV-specific immunity early after SHIV exposure was observed in the IFNγ ICS assay, but a blunted response was detected in the IFNγ ELISpot assay, concomitant with an increased granulocyte contamination of PBMC populations.

The increase in granulocytes within PBMC isolated by density gradient centrifugation during acute SHIV infection suggests that activation of granulocytes results in a density change causing granulocytes to co-purify with PBMC. Other groups have observed a similar phenomenon in human blood samples, both in vitro, by activating blood samples, and in vivo in patients with cancer or undergoing G-CSF treatment (Schmielau and Finn, 2001; Vasconcelos et al., 2003). Furthermore, one group have suggested that ex vivo activated human granulocytes can inhibit bulk cyto-

kine production from mitogen-activated T cells (Schmielau and Finn, 2001).

Potential mechanisms of the inhibition of spot formation by granulocytes could have involved either a generalized down-regulation of T cell IFNγ secretion (Schmielau and Finn, 2001), possibly via pro-inflammatory cytokines such as IL-10 and GM-CSF, or physical disruption of spot formation by migrating activated granulocytes. The latter explanation is preferred since blocking IL-10 and GM-CSF activity with antibody did not influence the quality of spots formed nor their number and microscopic evaluation of spots suggested that physical disruption of IFNγ spots was occurring. Furthermore, a dramatic improvement in spot quality was observed when an $F(ab)_2$ version of the capture antibody was used in place of full length antibody suggesting that spot disruption may have been caused by granulocyte activation through the Fc receptor. Granulocyte activation presumably enhances mobility and functional characteristics that physically interfere with capture of IFNγ released from T cells, possibly by increased adherence (Kapp et al., 1988). Future studies tracking fluorescent granulocytes at varying states of activation could more definitively resolve precise mechanisms on their inhibition of spot formation.

We believe that the problem of granulocytes may be even greater than our results indicate since spot disruption by granulocytes was not always associated with reduced spot counts. In some samples, disrupted spots were resolved as multiple spots by the automated ELISpot reader because of artificial contrast that is introduced, paradoxically resulting in an increase in spot number in these samples and minimizing the numerical differences reported.

In summary, we show the major effect of contaminating granulocytes on the function of the ELISpot assay, a critical assay used for HIV vaccine development. Activated granulocytes were more numerous during acute SHIV infection of macaques and profoundly reduced reliable enumeration of SIV-specific T cell numbers by ELISpot. We present at least a partial solution to this problem, using an $F(ab)_2$ rather than a full length capture antibody, which suggests that activation of granulocytes by the Fc portion of the capture antibody is a major determinant of this effect.

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