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Immune profiling of influenza-specific B cell and T cell responses in macaques using flow cytometry-based assays

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ABSTRACT

Influenza remains a significant global public health burden, despite substantial annual vaccination efforts against circulating virus strains. As a result, novel vaccine approaches are needed to generate long-lasting and universal broadly cross-reactive immunity against distinct influenza virus strains and subtypes. Several new vaccine candidates are currently under development and/or in clinical trials. The successful development of new vaccines requires testing in animal models, other than mice, which capture the complexity of the human immune system. Importantly, following vaccination or challenge, the assessment of adaptive immunity at the antigen-specific level is particularly informative. In this study, using peripheral blood mononuclear cells (PBMCs) from cynomolgus macaques, we describe detection methods and in-depth analyses of influenza virus-specific B cells by recombinant hemagglutinin probes and flow cytometry, as well as the detection of influenza virus-specific $CD8^+$ and $CD4^+$ T cells by stimulation with live influenza A virus and intracellular cytokine staining. We highlight the potential of these assays to be used with PBMCs from other macaque species, including rhesus macaques, pigtail macaques and African green monkeys. We also demonstrate the use of a human cytometric bead array kit in detecting inflammatory cytokines and chemokines from

cynomolgus macaques to assess cytokine/chemokine milieu. Overall, the detection of influenza virus-specific B cells and T cells, together with inflammatory responses, as described in our study, provides useful insights for evaluating novel influenza vaccines. Our data deciphering immune responses towards influenza viruses can be also adapted to understanding immunity to other infections or vaccination approaches in macaque models.

INTRODUCTION

Seasonal influenza A and B viruses (IAV and IBV) co-circulating annually as well as antigenically novel IAV sporadically emerging from animal reservoirs can cause devastating health and socio-economic impacts, despite annual vaccination efforts(1). Development of novel broadly cross-reactive and long-lasting influenza vaccines is essential for limiting the global impact of influenza viruses.

Such translational and clinical studies against influenza viruses, and other infectious diseases, greatly depend on animal models to generate proof-of-concept data for safety, efficacy and protection. However, many biological phenomena observed in humans cannot be recapitulated in mouse models, particularly for numerous infectious viruses such as HIV-1(2). In such cases, non-human primate (NHP) models of infection become an integral part of translational research since they can more effectively model human disease across a wide range of pathogens and capture the complexity of the human immune system(3). For instance, macaques have provided unique insights into influenza pathogenesis and vaccine assessment against human pandemic and avian influenza viruses(4).

The ability to track influenza virus-specific B and T cells, which mediate protection from infection and severe disease, can provide key insights into the cellular events underpinning generation of protective immunity following influenza virus infection or vaccination in NHP(5-8). However, there are only a handful of studies on influenza-specific B cells and a very limited literature on the detection of influenza virus-specific T cells in macaques. Here, we describe and validate two assays to quantify and characterize influenza virus-specific B cells and T cells in cynomolgus macaques and also demonstrate the use of commercially available

human cytokine detection methods for analysis of macaque samples. The use of these techniques enables dissection of immunity to influenza viruses at a great level of resolution, highly relevant also to other pathogens.

RESULTS

Detection of influenza virus-specific B cells in PBMCs from cynomolgus macaques.

Antigen-specific B cells are generally assessed by a combination of an *in vitro* polyclonal culture and ELISpot assays. However, recently the use of recombinant antigen protein B cell probes revolutionized our detection of antigen-specific B cells by direct *ex-vivo* flow cytometry(9-12). Using a similar approach on PBMCs from cynomolgus macaques, we established a panel of surface markers (Supplementary table 1), utilizing human antibody clones that cross-react with cynomolgus macaques to delineate a population of live $CD45^+CD3^-CD14^-CD16^-CD10^-CD19^+CD20^+$ B cells (Fig 1a). We excluded any PBMCs non-specifically reacting to free streptavidin-BV510 conjugates. Within B cells, IgD^+ non-class switched B cells were also excluded as they exhibit a high degree of non-specific binding(9). Within the IgD^- B cells, a substantial population of pdm09 H1N1 virus (A/California/7/2009) recombinant (r) HA-PE-specific B cells could be readily detected in an animal previously infected with pdm09 H1N1 A/California/7/2009 (Fig 1a). Our panels further allowed isotype profiling analyses with respect to IgG and IgM on class-switched and rHA-specific B cells and showed that rHA-PE-specific B cells in H1N1 A/California/7/2009 virus-infected monkeys were predominantly of the IgG isotype.

Although we and others have previously validated the specificity of these probes in human samples(9-12), here we further verified the specificity of rHA probes in macaque PBMCs in two ways. Firstly, we analyzed PBMC from 21 influenza-naïve animals with 20/21 animals having undetectable frequencies of H1-rHA^+ B cells (Fig 1b; <0.05%, mean 0.027% of IgD^- B cells). Of these animals, 3 were then intranasally challenged with a pre-pandemic seasonal influenza virus strain, A/Yokohama/91/2007 (H1N1). On day 56 after infection, only negligible frequencies of H1-rHA^+ B cells were detected (mean 0.027%), which overall were

not statistically higher than the naïve group. Any H1-rHA$^+$ B cells in this group could represent rare cross-reactive B cells targeting conserved epitopes in this antigenically distant H1 protein. The three animals were then re-infected with a heterologous H1N1pdm09 strain (A/Narita/1/2009). On day 8 after infection, significantly higher frequencies of H1-rHA$^+$ B cells were detected (mean 0.38%, *P* <0.0001). To validate rHA probe specificity, we co-stained samples from the last timepoint, with the same rHA probe on two different fluorochromes (Fig 1c; PE and APC). Double staining of antigen-specific B and T cells with the cognate probe or tetramers on two different fluorochromes is a well-established method to confirm the specificity of staining. This approach differentiates probe-specific staining (double-stained cells) from possible background staining (single-stained events)(13). The majority (87.2%) of rHA$^+$ B cells were positive for both fluorochromes, indicating a high degree of specificity. Thus, as shown by analysis of PBMCs from naïve animals and animals infected with an antigenically-distant or an antigenically-related strain as well as by dual rHA staining, flow-cytometry-based staining with rHA probes enable accurate detection of antigen-specific B cells in macaques.

Detection of influenza virus-specific CD4$^+$ and CD8$^+$ T cells in PBMCs from cynomolgus macaques.

We next assessed influenza virus-specific CD4$^+$ and CD8$^+$ T cells, adopting an assay previously used on human PBMCs to assess immune response against IAV and IBV(9, 14). Cryopreserved PBMCs from influenza A/Narita/1/2009(H1N1)-infected cynomolgus macaques were stimulated with live influenza A virus (A/Singapore/GP1908/2015 (IVR-180) (H1N1) pdm09) overnight in the presence of the Brefeldin A to assess IFNγ and TNF production in CD4$^+$ and CD8$^+$ T cells by intracellular cytokine staining using flow cytometry (Figure 2a, Supplementary table 2). CD4$^+$ and CD8$^+$ T cells were analyzed as single live CD4$^+$CD8$^-$CD3$^+$ and CD4$^-$CD8$^+$CD3$^+$, respectively (Fig 2b). Within those two subsets, IFNγ and TNF production was readily detected in IAV-stimulated but not mock-treated samples, nor in influenza virus-stimulated PBMCs from an influenza-naïve animal. We assessed infection rates of monocytes in PBMCs, cell viability as well as IFNγ production after infection at a multiplicity of infection (MOI) of 4 and 6 (Fig 3). Monocyte infection rates were modestly but significantly higher (36% versus 40%, *P*=0.039) at an MOI of 6 when compared to MOI of 4, without compromising viability (83% across all

conditions, *P*=0.99) (Fig 3a, b). IFNγ production in both $CD4^+$ and $CD8^+$ T cells was readily detectable at an MOI of 4 and 6, and was largely similar across both MOI in the 3 samples tested (Fig 3c). Overall, our study shows that this 18-hr IFN-γ assay previously developed for human PBMCs, can be also used to detect influenza virus-specific $CD4^+$ and $CD8^+$ T cells within the PBMCs of influenza virus-exposed macaques.

Applicability of flow cytometry panels to other non-human primate species.

As cynomolgus macaques are not the only NHP model used for translational research (2-4), we assessed the applicability of our flow cytometric panels in other species commonly used in clinical trials, including pigtail macaques (*Macaca nemestrina*), rhesus macaques (*Macaca mulatta)* and African green monkeys (*Chlorocebus sabaeu*), which are also used as infection models for influenza viruses (4, 6-8), HIV/SIV(2), flaviviruses (15), Ebolaviruses(16) and coronaviruses(17). Based on the NIH NHP Reagent Resource(18), the Biolegend Antibody Cross-reactivity Chart(19), and previous studies(8, 20, 21), all but one antibody clone in our panels can be used in all of 4 species (Supplementary table 3). While the anti-CD4 clone OKT4 is not cross-reactive with the CD4 molecule of African green monkeys(18), it can be readily replaced by other clones like L200 or SK3 (Leu-3A) that cross-react across all 4 species(18). Thus, the panels and assays described here are applicable to at least 4 species of non-human primates, commonly used for translational research and clinical trials.

Detection of cytokines and chemokines in influenza virus-infected cynomolgus macaques by cytometric bead array

In addition to the analysis of antigen-specific adaptive immunity, understanding innate immunity and inflammation is essential for assessing reactogenicity and adverse events, and measuring disease severity (eg. cytokine storm in severe respiratory infections)(22). Multiple cytokines can be readily measured using multiplexed cytometric bead array (CBA) kits, commonly used for human and mouse samples. While NHP specialized kits for such experiments are available, we determined the potential of human reagents to cross-react with macaque cytokines, as these are a useful tool when NHP kits are not available or for pilot studies prior to

the purchase of NHP specialized kits and their costs. To that end, PBMCs were stimulated with PHA for 24 hours to induce cytokine and chemokine production which were measured in the culture supernatant. Using a human CBA kit for 17 analytes (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, RANTES, MCP-1, MIP-1α, MIP-1β, TNF, IFNα, IFNγ, FasL and Granzyme B), we could readily detect 10/17 cytokines/chemokines at concentrations higher than unstimulated cells (Fig 4a). IFNα, RANTES, IFNγ, Granzyme B, CD178 (FasL), IL-10 and IL-12p70 were variably detectable or undetectable and did not increase following stimulation. It is unclear whether these are not produced following PHA stimulation or are produced but the human reagents are not cross-reactive. The detection of these 7 cytokines/chemokines using the human CBA kit warrants further investigation. To further assess the use of the human reagents on macaque samples, we analysed serum samples from 18 animals challenged with A/Yokohama/91/2007 (H1N1) intranasally and sampled at 6 and 24 hours after infection. At these very early timepoints, we could detect RANTES, IL-8, MCP-1 and IL-6 (Fig 4b). Compared to baseline, the levels of IL-6 and IL-8 significantly (P<0.02) increased at 6 and/or 24 hours after infection. There was also a trend towards increased RANTES and MCP-1 at those time points, albeit with greater heterogeneity. The lack of detection of the other cytokines/chemokines was likely due to differences in kinetics of expression, compartmentalized induction at the site of infection and/or the presence of these analytes in serum below the limit of cross-detection by the human CBA kit under these experimental conditions. Nonetheless, our analysis demonstrates the potential applicability of a human CBA kit for the assessment of inflammatory mediators *in vitro* as well as *ex vivo,* useful in pre-clinical and clinical trials.

DISCUSSION

The analysis of antigen-specific B cell and T cell immunity is an essential component of any clinical trial study as it provides an indication of the strength and longevity of induced immune responses following vaccination and infection. While well-established methods to achieve that exist for murine systems as well as human samples, such methods have been less developed for NHP. Here, we describe novel flow-cytometric analyses of influenza-specific B cells and T cells in cynomolgus macaque samples and highlight their potential to be used in samples from other commonly used macaque species.

The ability to track antigen-specific B cells by rHA probes and flow cytometry directly *ex vivo*, as opposed to polyclonal expansion and ELISpot assays, can allow for the rapid quantification of B cell numbers and the accurate phenotyping of these population without the confounding effects of *in vitro* activation. The application of such techniques on human samples has provided novel insights into the breadth of the antibody response(23) and cellular events underpinning generation of effective immunity(9). In NHP, such techniques can provide new insights into the B cell response to influenza vaccination and infection(6, 8). Here, we established a flow cytometric panel that can accommodate probes of two different antigens and validated the specificity of H1 rHA probes. Since similar recombinant protein probes are used to track B cells specific to different pathogens, including HIV-1(24), malaria(25), Zikavirus(26), RSV(27) and SARS-CoV-2(28), this assay has applications beyond the influenza field.

As T cells recognize MHC molecules in complex with virus-derived peptides(29), they can be readily tracked by fluorescently-labelled peptide-MHC tetramers. However, this requires (i) species-specific reagents, (ii) knowledge of immunogenic peptides, and (iii) MHC typing of the experimental animals, with the latter two requiring coverage across the various MHC allotypes found within outbred populations of the same species(5, 30). Here, we describe an assay that allows for the detection of influenza-specific $CD4^+$ and $CD8^+$ T cells from a relatively small number of PBMC directly *ex vivo*, based on IFNγ and TNF production, irrespective of MHC allotypes and without knowledge of immunogenic proteins or peptides. The antibody panel used in this assay is applicable to other NHP species and the principle of the assay should therefore be readily applied to other species and other viruses, although may require optimization due to differences in tropism and infectivity across different viruses.

Lastly, we demonstrate that commercial kits for human cytokine analysis can be utilized for the detection of inflammatory cytokines and chemokines in cynomolgus macaques. Although the specificity and sensitivity of the human reagents for some of the cytokines/chemokines needs to be assessed further, our analysis suggests that these reagents may be useful in analysing innate immunity to influenza virus and the potential reactogenicity of novel vaccines.

Overall, our study defines novel and broadly applicable assays for the antigen-specific analysis of B and T cells in NHP that will aid the development of effective therapeutics.

METHODS

Animals

This study was approved by the Institutional Animal Care and Use Committee (Approved number IACUC435-001 and IACUC435-002) and was performed in accordance with the animal welfare by laws of SNBL, which is accredited by American Association for Accreditation of Laboratory Animal Care (AAALAC) International. Healthy 11 female and 10 male cynomolgus monkeys (*Macaca fascicularis*) weighing 2-3.5 kg and aged 2-3 years old, were purpose-bred in Cambodia and imported and maintained at Shin Nippon Biomedical Laboratories (SNBL). For influenza challenged models, three macaques were intranasally challenged with 4×10^5 $TCID_{50}$ mL^{-1} of pre-pandemic strain of influenza A/Yokohama/91/2007 (H1N1) and re-challenged with 2×10^5 $TCID_{50}$ mL^{-1} of 2009 pandemic strain of influenza A/Narita/1/2009 (H1N1) at day 77. Blood samples were collected in heparin tubes from the femoral vein at day 0 (included 6 hours and 24 hours post challenge), 56 and 85. Blood samples were also collected from an additional 18 naïve macaques. Serum was separated and stored at -80^{o}C until use.

For experiments in Fig 4, whole blood samples were collected from adult cynomolgus macaques (*Macaca fascicularis*, Monash Animal Research Platform) and for staining in Sup Fig 1, whole blood samples were collected from juvenile pigtail macaques (*Macaca nemestrina*, Australian National Breeding Colony). All procedures were approved by Monash University Animal Ethics Committee (#19372) or Australian Commonwealth Scientific and Industrial Research Organization Animal Health Animal Ethics Committee (#1656). PBMCs were isolated by Ficoll gradient (95% Ficoll), cryopreserved in 90% fetal calf serum/10% DMSO, and stored in liquid nitrogen until analysis.

Viruses

Influenza A (A/Singapore/GP1908/2015(IVR-180) (H1N1) pdm09, A/Yokohama/91/2007 (H1N1), A/Narita/1/2009 (H1N1) pdm09) viruses were grown in 10-day old embryonated chicken eggs at 35^{o}C for 48 hours. Allantoic fluid was harvested and titrated using standard plaque assays in MDCK cells.

rHA staining of PBMC

rHA probes specific for the A/California/7/2009 H1N1pdm09 HA were generated and used for staining HA-specific B cells as described(9, 11). Thawed PBMCs were stained with antibodies in Supplementary table 1. Staining with rHA probes was performed at the same time as cell surface staining at 4^{o}C for 30 mins in 1% fetal bovine serum (FBS)/PBS. Cells were then fixed in 1% PFA for flow cytometry.

Inflection of PBMCs with influenza viruses and intracellular cytokine staining

Stimulation of macaque PBMCs with live influenza viruses was performed as previously described for human PBMCs(9, 14). Briefly, cryopreserved PBMCs were thawed at 37^{o}C in serum-free RPMI media (RPMI 1640; Thermo Fisher Scientific, MA, USA) supplemented with 1 mM of sodium pyruvate (Thermo Fisher Scientific), 50 mM of 2-mercaptoethanol (Merck, Darmstadt, Germany), 100 mg mL^{-1} of penicillin (Thermo Fisher Scientific), 100 mg mL^{-1} of streptomycin (Thermo Fisher Scientific), and 20 mg mL^{-1} of gentamicin (Thermo Fisher Scientific). Approximately 10^{6} PBMC were infected with live influenza A virus at a MOI of 6 or with media alone (mock), in serum-free media at 37^{o}C. After 1 hour, FBS was added at a final concentration of 10% and the cells were incubated for 3 hours at 37^{o}C. At that time, Golgi Plug (BD, NJ, USA) was added at a final concertation of 1µg mL^{-1} to inhibit protein export and the cells were incubated at 37^{o}C for 16 hours. After a total of 20 hours after infection, cells were harvested and stained with surface antibodies (Supplementary table 2) in 50µl of stain buffer (1% FBS–5mM EDTA–PBS) for 30 mins at 4^{o}C. Cells were washed and then fixed and permeabilized in 100µl BD CytoFix/CytoPerm for 20 mins. After washing, lymphocytes were stained with intracellular antibodies (Supplementary table 2) in 50 µL of BD Perm/Wash buffer for 30 mins at 4^{o}C, prior to acquisition in a BD Fortessa.

Cytokine measurement by cytometric bead array

PBMC (2 x10^6) were stimulated with PHA (5 µg mL-^{1}) for 24 hours in RF10 media (RPMI 1640; Thermo Fisher Scientific) supplemented with 10% inactivated fetal bovine serum (FBS; GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK), 1 mM of sodium pyruvate (Thermo Fisher Scientific), 50 mM of 2-mercaptoethanol (Merck), 100 mg mL-^{1}of penicillin (Thermo Fisher Scientific), 100 mg mL-^{1} of streptomycin (Thermo Fisher Scientific), and 20 mg mL-^{1} of gentamicin (Thermo Fisher Scientific). The culture supernatant was collected and stored at -20^{o}C until further use. Culture supernatant and serum samples (diluted 1:4) from infected macaques (obtained at 0, 6 and 24 hours after infection) were analyzed using a BD Human Cytometric Bead Array kit (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, RANTES, MCP-1, MIP-1α, MIP-1β, TNF, IFNα, IFNγ, FasL and Granzyme B), according to the manufacturer's instructions, as previously described(31) and acquired on a BD Canto II.

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FIGURE LEGENDS

Figure 1. Detection of influenza virus-specific B cells in PBMCs from cynomolgus macaques. **(a)** Gating strategy for the identification of influenza virus-specific B cells. B cells were identified as $CD19^+CD20^+$ cells within live $CD3^-CD14^-CD16^-CD10^-$ events. Influenza virus -specific B cells were identified as rHA^+ within class-switched (IgD^-) B cells. Total and rHA^+ B cells are further characterized based on their expression of IgG and IgM. **(b)** Detection of H1N1pdm09 virus-specific B cells in naïve (n=21), H1N1-Yokohoma primed macaques (n=3) or H1N1-Yokohoma virus primed/H1N1-Narita virus boosted macaques (n=3). Representative FACS plots are shown for each group. **(c)** Validation of specificity of the H1N1pdm09 rHA probe by double staining using the same rHA probe on two different fluorochromes (APC and PE). Representative FACS plots are shown. Mean and SD are shown from one experiment. Statistical significance comparing all groups with each other

was determined by a one-way ANOVA with Turkey's correction for multiple comparisons. Only statistically significant comparisons are indicated on the graph.

Figure 2. Detection of influenza virus-specific $CD4^+$ and $CD8^+$ T cells in PBMCs from Cynomolgus macaques. (a) Overview of methodology. ~10^6 cryopreserved PBMCs were infected with live influenza A virus at a multiplicity of infection (MOI) of 6 in serum-free media at 37°C. After 1 hour, fetal calf serum (FCS) was added at a final concentration of 10% and PBMCs were incubated for 3 hours at 37°C. At that time, Brefeldin A (BFA) was added to inhibit protein export and the cells were incubated at 37°C for 16 hours. Subsequently, PBMCs were harvested, fixed and stained for surface and intracellular markers prior to analysis by flow cytometry. **(b)** Gating strategy for the identification of influenza-specific $CD4^+$ and $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells were identified as $CD3^+CD4^+$ or $CD3^+CD8^+$ cells within live ingle lymphocytes, excluding double-positive $CD4^+CD8^+$ events. Within CD4 or CD8 T cells, IFNγ and TNF production was measured in IAV-stimulated cells compared to mock-stimulated cells. Data from one experiment.

Figure 3. Optimization of influenza virus-specific T cell detection in macaque PBMCs. (a-b) Assessment of different multiplicities of infection (MOI) on infection rate **(a)** and cell viability **(b)**. **(a)** Macaque PBMCs were infected at a MOI of 4 or 6 and the frequency of IAV nucleoprotein-positive (NP^+) monocytes (identified based on FSC-A and SSC-A) was quantified (n=9, from two independent experiments). **(b)** Macaque PBMC were infected at MOI of 4 or 6 and the frequency of live cells was quantified (n=4-9, from 1 or 2 experiments). **(c)** Frequency of $IFN\gamma^+$ $CD4^+$ (top panel) and $CD8^+$ (bottom panel) T cells at different MOI (n=3). Data from one experiment. Statistical significance comparing all groups with each other was determined by a one-way ANOVA with Turkey's correction for multiple comparisons. Only statistically significant comparisons are indicated on the graphs.

Figure 4. Detection of macaque cytokines and chemokines using a human CBA kit. (a) Assessment of 17 cytokines and chemokines in the supernatant of PBMC stimulated with PHA for 24 hours. Data from two cultures using PBMCs from two different animals are shown. **(b)** Detection of cytokines and chemokines in the

serum of macaques at 0, 6 and 24 hours after infection with A/Yokohama/91/2007 (H1N1) virus. All 17 analytes were assessed but only the detectable ones are shown (n=18 animals). Statistical significance comparing all groups with each other was determined by a Kruskal-Wallis with Dunn's correction for multiple comparisons. Data from one experiment.

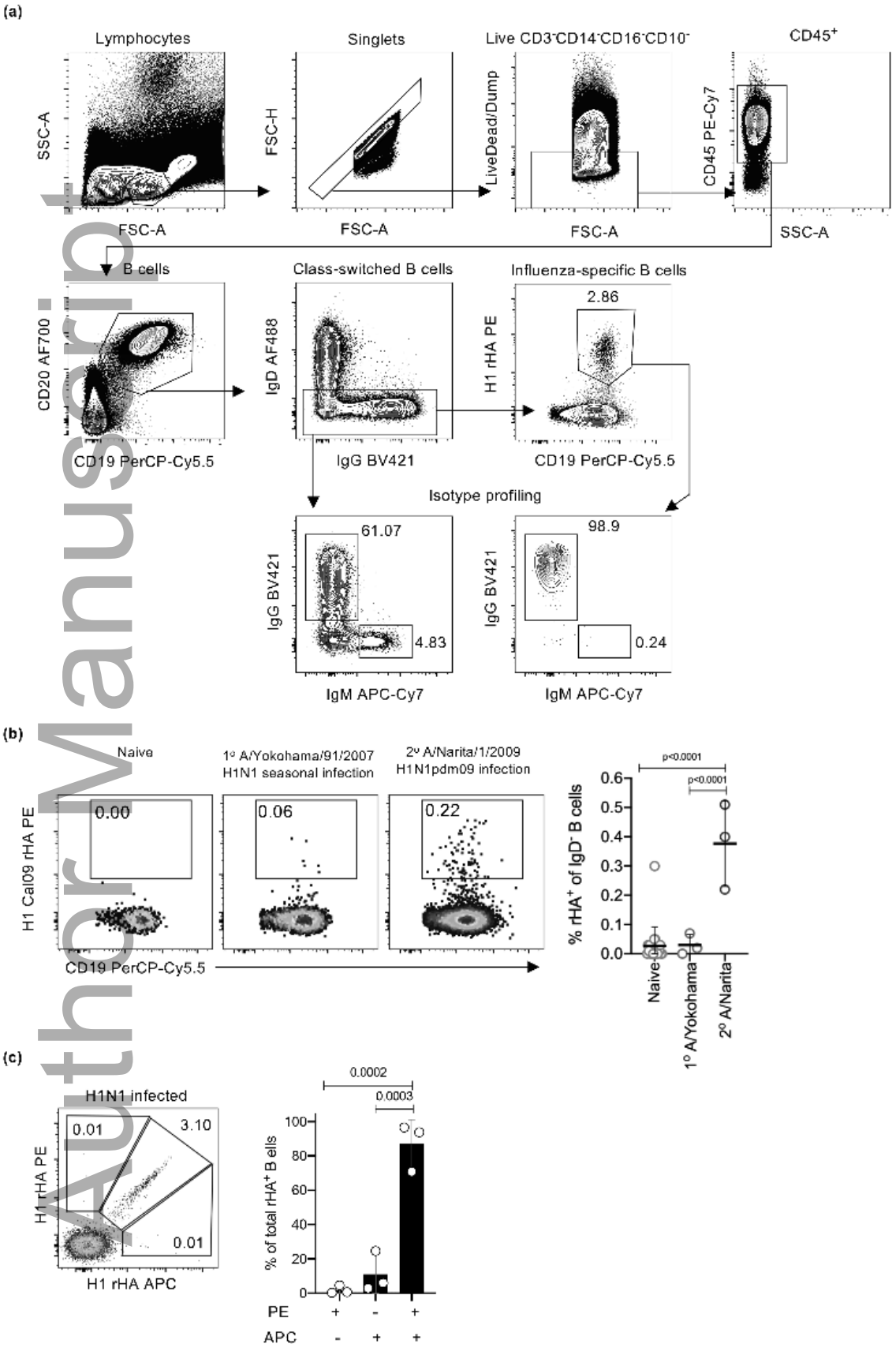

Figure 1. Koutsakos M*, Sekiya T* *et al*

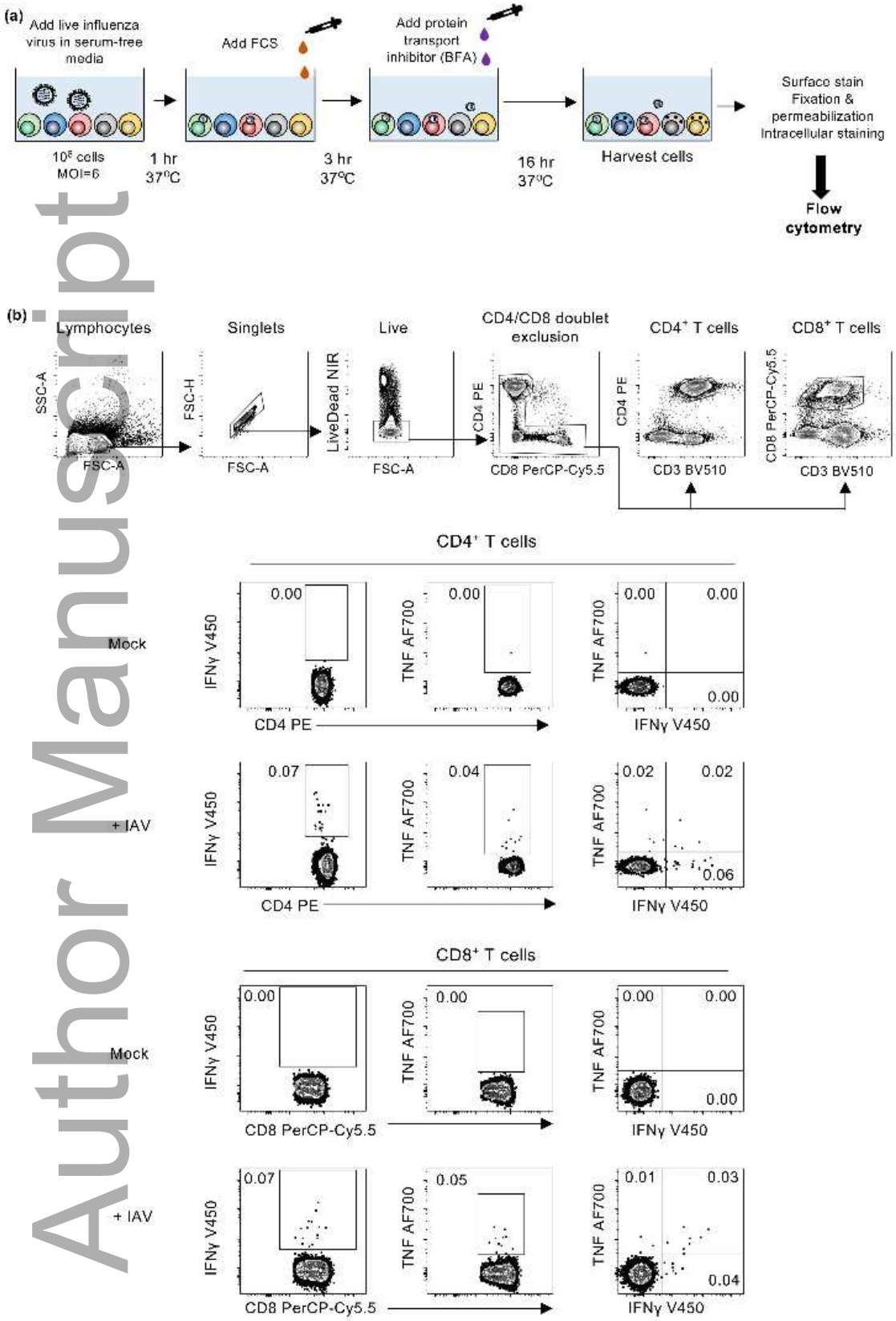

Figure 2. Koutsakos M*, Sekiya T* *et al*

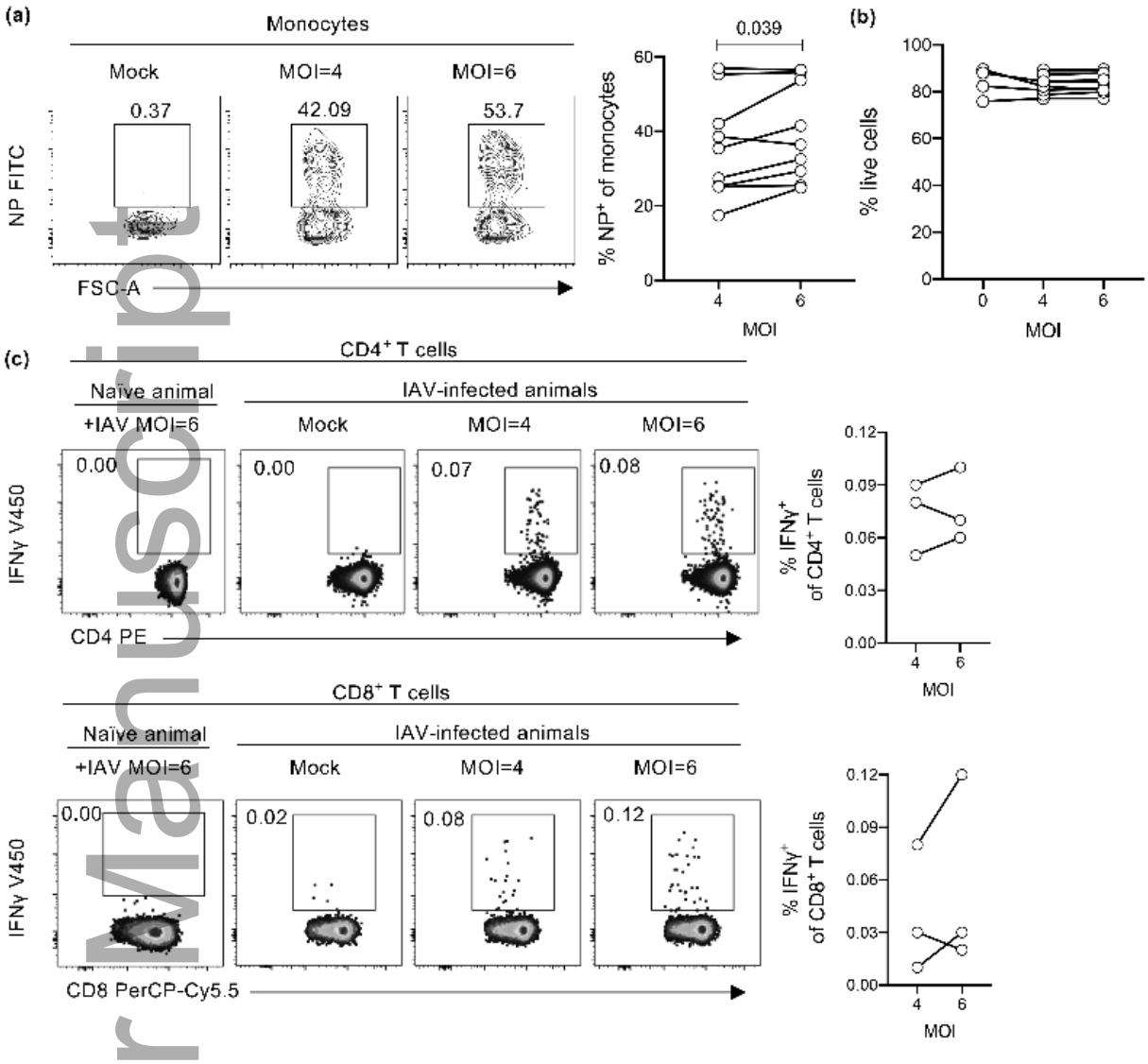

Figure 3. Koutsakos M*, Sekiya T* *et al*

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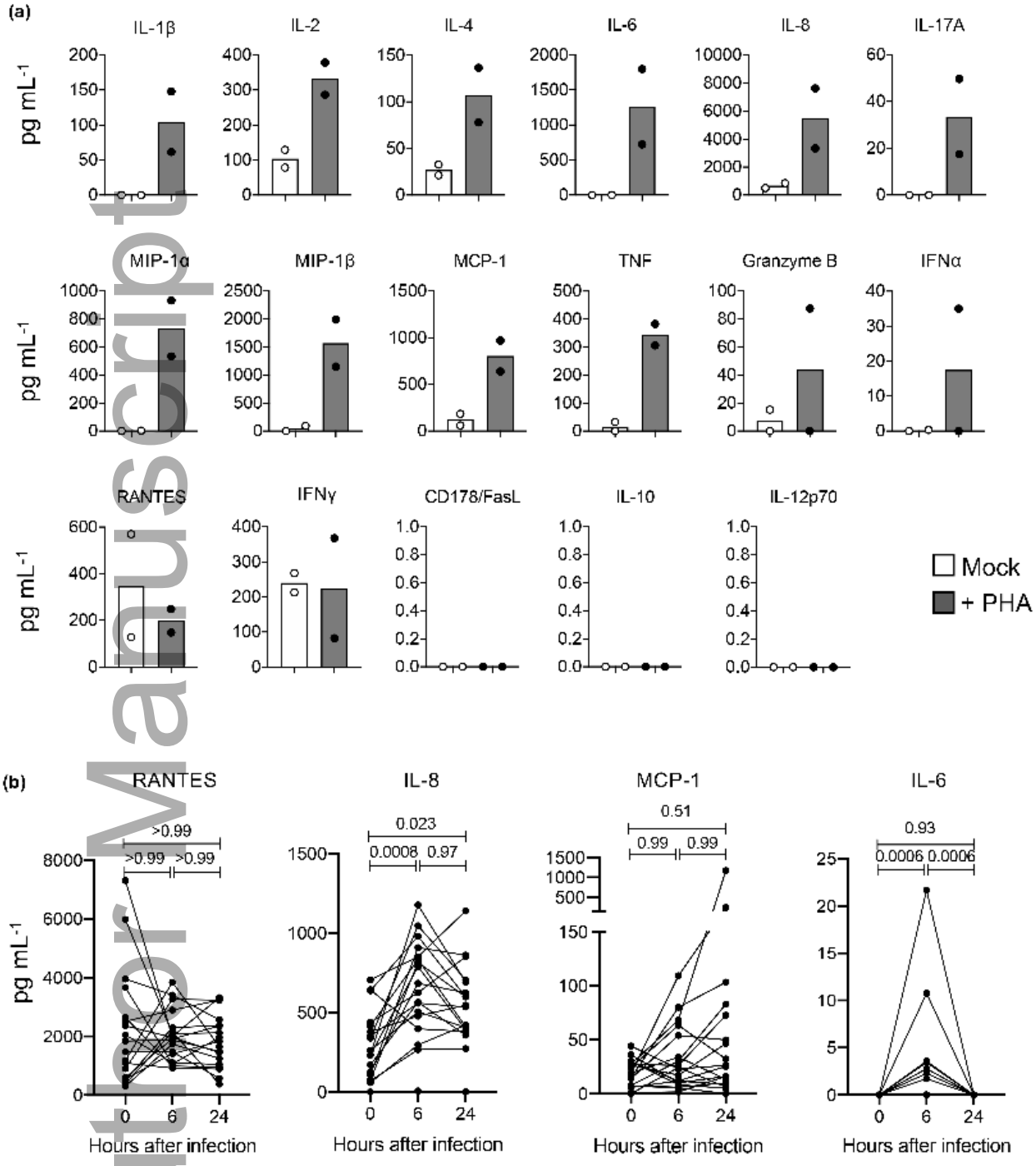

Figure 4. Koutsakos M*, Sekiya T* *et al*

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