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Technical note

T cell acquisition of APC membrane can impact interpretation of adoptive transfer experiments using CD45 congenic mouse strains

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Abstract

Congenic mouse strains bearing allelic variants of CD45 are often used in adoptive transfer experiments. Here, we report that immune $CD8^+$ splenocytes of donor origin acquired the recipient's congenic CD45 marker during interaction with antigen-bearing cells, presumably as a result of membrane transfer upon dissolution of the immunological synapse. Acquisition of recipient marker by donor cells was most prominent after in vitro incubation with peptide for intracellular cytokine staining, where most of the antigen-bearing splenocytes are of recipient origin. In consequence, when antibodies against the recipient's congenic marker were used to distinguish donor and recipient populations, donor origin cells were incorrectly interpreted as being of recipient origin. This phenomenon may cause problems for interpretation of data in adoptive transfer experiments primarily when (a) staining for the recipient's congenic marker and (b) identifying antigen-specific populations by staining for intracellular cytokine.

Keywords: Membrane transfer; Adoptive transfer; CD45 congenic; Murine cytomegalovirus

1 . Introduction

Identifying and isolating cellular components of the adaptive immune response are important steps for understanding the complex interplay among various lymphocyte subsets during an immune response. A common technique for this purpose is the use of adoptive transfer experiments between congenic strains of mice. Congenic markers, such as CD45.1 and CD45.2, can then be used to distinguish donor and recipient cells. Most commonly, the experimental goal requires tracking the donor cell population. Sometimes, however, the impact of the transferred cells on the recipient's immune response is of interest, and this necessitates accurate identification of the recipient cell populations. We were interested in understanding the impact of transferred memory CD8 T cells on the primary immune response to murine cytomegalovirus (MCMV). We identified antigen-specific CD8 T cells by intracellular cytokine staining (ICS), and attempted to identify the response of the naïve recipient cells by staining for the recipient's CD45 congenic marker. During these experiments, we realized that donor cells had come to express the recipient's CD45 congenic marker, presumably due to acquisition of recipient cell membrane after antigen recognition. This caused a serious misinterpretation of the data when we used the recipient's congenic marker in attempt to identify the recipient T cell population.

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2. Materials and methods

2.1. Mouse and virus strain

Mice congenic in CD45 at the Ly5 locus (C57BL/6J-Ly5.2 (CD45.2) and B.6SJL-Ptprca Pepcb/BoyJ-Ly5.1 (CD45.1)) were purchased from The Jackson Laboratory. Mice, aged −21–23 weeks, were intraperitoneally infected with 2×10^6 pfu MCMV strain MW97.01 which was derived from a bacterial artificial chromosome of Smith strain MCMV (Wagner et al., 1999). These mice were latently infected for approximately 6–16 weeks and were used as a source of transferred splenocytes. Uninfected congenic recipient mice were aged 12–18 weeks. Mice were housed at Oregon Health and Science University and all studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

2.2. Surface and intracellular cytokine staining

Approximately 10^6 splenocytes were surface stained with the following antibodies: anti-CD8α Pacific Blue (53–6.7, eBioscience) at 1/300 dilution, anti-CD45.1 FITC (A20) at 1/300 dilution, anti-CD45.1 PE and PE-Cy7 (A20, eBioscience) both at 1/100 dilution, anti-CD45.2 PE (clone 104, eBioscience) at 1/100 dilution, anti-CD45.2 APC-Cy7 (clone 104, eBioscience) at 1:50 dilution and anti-CD45.2 APC (clone 104, eBioscience) at 1/300 dilution. The anti-CD45.1 antibody was isolated from hybridoma culture supernatant and conjugated to FITC (Sigma-Aldrich) using a standard conjugation protocol with cross-linker, SMCC (succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate, Pierce). M38-K^b tetramer was used at 1/600 dilution and was generated from the NIH Tetramer Facility. Staining with M38 tetramer was done for 1 h on ice. Staining for CD8α, CD45.1 and CD45.2 was performed overnight at 4 °C.

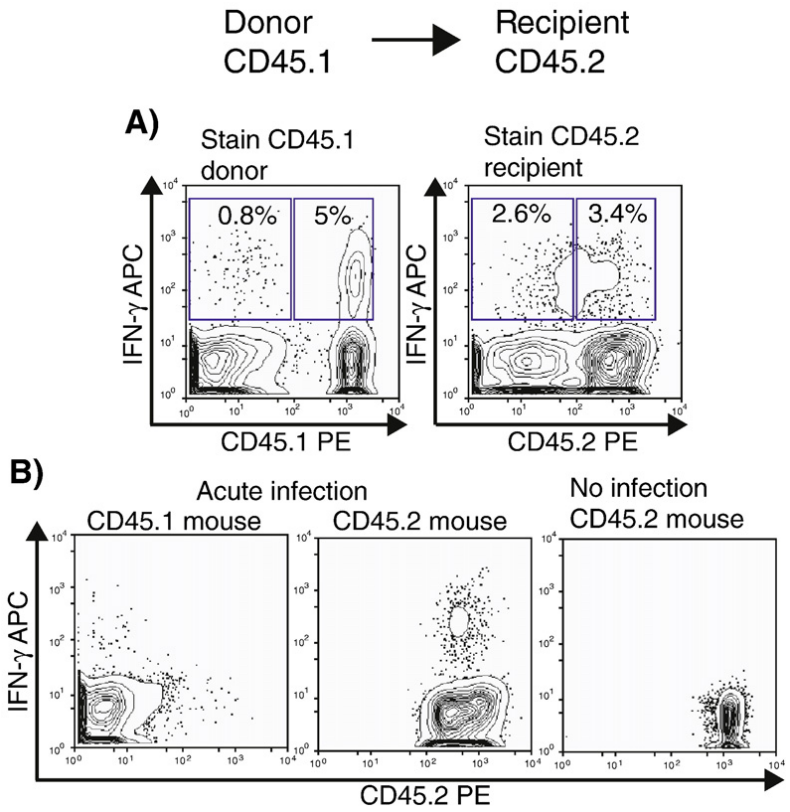

Fig. 1. Disparate determination of recipient response when staining for donor versus recipient congenic marker. Splenocytes from a latently MCMV-infected CD45.1 mouse were transferred to a naïve CD45.2 recipient, which was inoculated with MCMV the next day. One week later, splenocytes were stimulated with M38 peptide followed by ICS for IFN-γ and staining for CD8 and CD45.1 or CD45.2. FACS plots shown are from an initial $CD8\alpha^+$ gate. A) Comparison of staining for donor CD45.1 marker (left panel) or recipient CD45.2 marker (right panel). B) CD8 T cell response from infected CD45.1 and CD45.2 mice without adoptive transfer and naïve CD45.2 control mouse.

For the intracellular cytokine staining, 10^6 splenocytes were in vitro stimulated with 1 μM M38 peptide (GeneMed) for 6 h in the presence of brefeldin A (Munks et al., 2006). Cells were washed twice in PBS containing 2% FBS and 0.1% azide (FACS buffer) and stained for cell surface molecules as listed above. The production of IFN-γ was detected using anti-IFN-γ PE (XMG1.2, Biolegend) or anti-IFN-γ APC (XMG1.2, eBioscience) antibodies with the Cytofix/Cytoperm kit (BD Pharmingen) according to manufacturer's instructions. Specifically, cells were washed twice with FACS buffer and fixed for 30 min in Cytofix reagent. Cells were then stained for IFN-γ in Cytoperm reagent for 30 min on ice and then washed twice in Cytoperm reagent. Samples were acquired on FACS Calibur (BD Pharmingen) with CellQuest software and analyzed with FlowJo software (Tree Star).

2.3. Adoptive transfer

Splenocytes from latently infected mice ranging from 3×10^7 to 10^8 cells, depending on the transfer experiment, were intravenously injected into CD45 congenic recipients. One day after transfer, recipient mice were intraperitoneally infected with 2×10^6 pfu MCMV. One week later, splenocytes from recipient mice were stained with antibodies as listed above and M38 tetramer directly ex vivo or for IFN-γ after in vitro peptide stimulation.

3. Results and discussion

To determine how pre-existing immunity would impact the immunodominance pattern of the response of naïve T cells to MCMV infection, splenocytes from latently infected CD45.1 congenic mice were transferred into naïve C57BL/6 mice (CD45.2) recipients. The recipients were then infected with MCMV and the CD8 T cell response to a panel of MCMV epitopes was assessed one week later by ICS. Using a standard protocol, splenocytes were stimulated with antigenic peptide for 6 h then stained with antibodies against CD8α and CD45.1 or CD45.2, followed by permeabilization and staining for IFN-γ. Because our experimental purpose was to study the recipient's response, we wanted to positively identify cells of recipient origin by staining for CD45.2. As seen in a representative FACS plot (Fig. 1A), the results of the ICS were difficult to interpret. When we stained for the recipient congenic marker (CD45.2), most antigen-specific cells displayed a CD45.2-intermediate phenotype, making it difficult to define clear recipient and donor populations. A conservative gate for CD45.2 suggested that 3.4% of $CD8^+$ cells were IFN-γ positive cells of recipient origin. However, when staining for the donor CD45.1 congenic marker only 0.8% of CD8 T cells were identified as CD45.1-negative, recipient-derived cells.

A possible explanation for the CD45.2-intermediate phenotype of IFN-γ positive cells seen in Fig. 1A could be that all the positive cells were in fact of recipient origin, but that CD45.2 expression is markedly decreased upon T cell activation. To assess this, we infected a CD45.2 mouse, without any adoptive transfer, as a control in this experiment (Fig. 1B, middle panel). Splenocytes from this mouse were assessed by ICS as in Fig. 1A. Comparison with a naïve mouse (Fig. 1B, right panel) showed that there was a modest level of CD45.2 downregulation after infection, and that the IFN-γ^+ cells had a slightly lower level of CD45.2 than cells from naïve mice. However, this decrease was minor and cells were clearly within the CD45.2 positive gate. Thus, CD45 downregulation upon activation did not account for the apparent CD45.2-intermediate phenotype seen in Fig. 1A.

We wanted to understand why staining for donor and recipient CD45 markers gave such discordant results. We wondered whether the problem might lie in the specific antibodies (anti-CD45.1 or anti-CD45.2), or problems with compensation between fluorescence channels during FACS analysis. We therefore performed the following experiment. Donor cells from either CD45.1 or CD45.2 congenic mice were transferred into CD45.2 or CD45.1 congenic recipients, respectively. Mice were then infected with MCMV and the CD8 T cell response analyzed by ICS one week later. For each transfer combination, recipient and donor cells were distinguished by staining for either the donor or the recipient's congenic marker. Additionally, three different fluorochrome combinations were used for each CD45.1 and CD45.2 recipient to investigate possible compensation issues. The results are shown in Fig. 2. In both transfer conditions, staining for the donor cells clearly separated donor and recipient populations and suggested that the vast majority of IFN-γ^+ cells were of donor origin. However, staining for the recipient's congenic marker gave quite a different pattern. As initially shown in Fig. 1, the CD45 staining of IFN-γ^+ cells was of intermediate intensity making it difficult to clearly discriminate two populations. Regardless of which fluorochrome combination was used, the mean fluorescence of the CD45-negative (presumed donor origin) IFN-γ^+ cells was shifted towards the right. The same shift did not occur in acutely infected (non-transfer) mice bearing the donor congenic marker; nor did it occur when cells from a naïve mouse were mixed

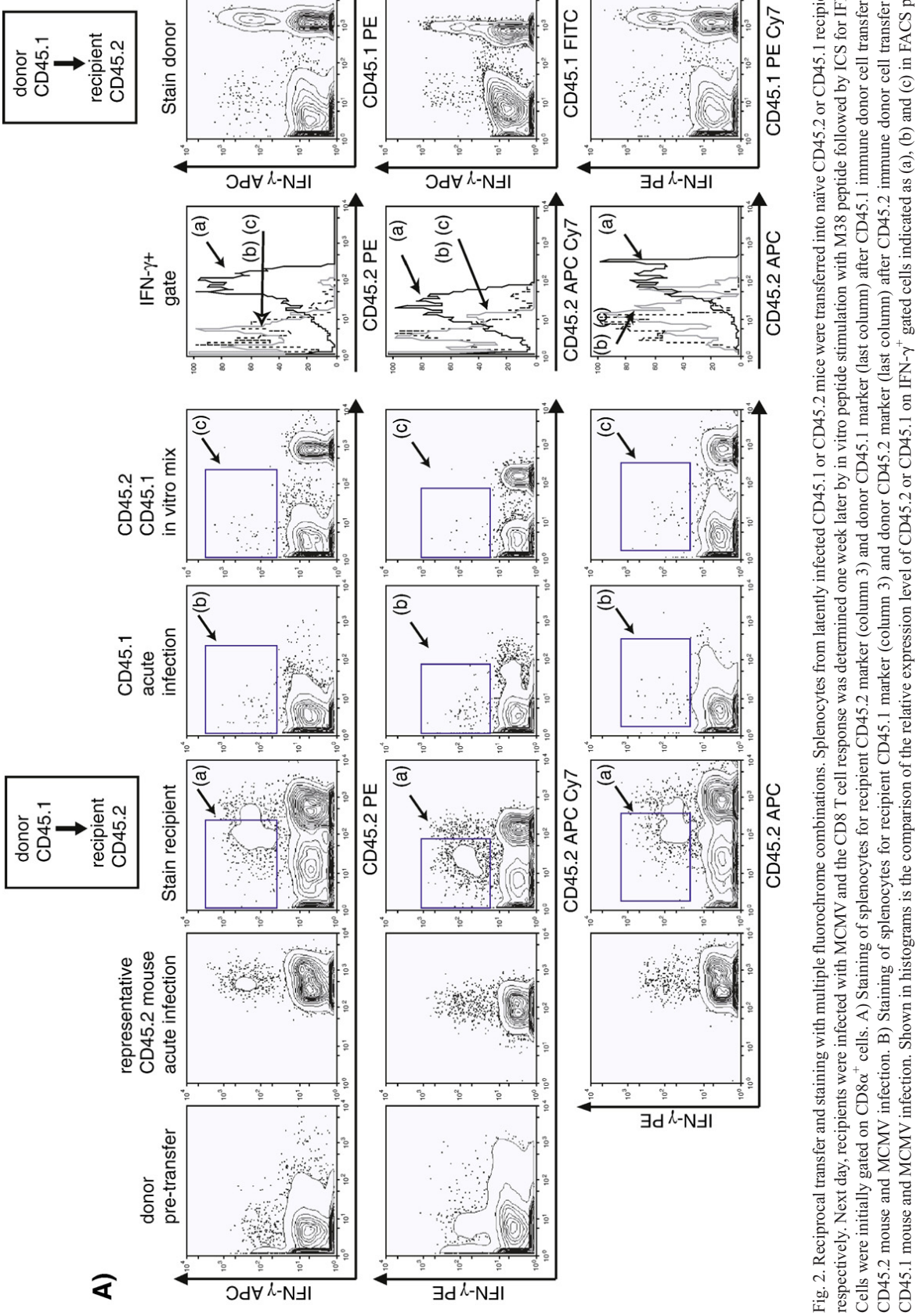

Fig. 2. Reciprocal transfer and staining with multiple fluorochrome combinations. Splenocytes from latently infected CD45.1 or CD45.2 mice were transferred into naïve CD45.2 or CD45.1 recipients, respectively. Next day, recipients were infected with MCMV and the CD8 T cell response was determined one week later by in vitro peptide stimulation with M38 peptide followed by ICS for IFN-γ. Cells were initially gated on CD8α$^+$ cells. A) Staining of splenocytes for recipient CD45.2 marker (column 3) and donor CD45.1 marker (last column) after CD45.1 immune donor cell transfer into CD45.2 mouse and MCMV infection. B) Staining of splenocytes for recipient CD45.1 marker (column 3) and donor CD45.2 marker (last column) after CD45.2 immune donor cell transfer into CD45.1 mouse and MCMV infection. Shown in histograms is the comparison of the relative expression level of CD45.2 or CD45.1 on IFN-γ$^+$ gated cells indicated as (a), (b) and (c) in FACS plots.

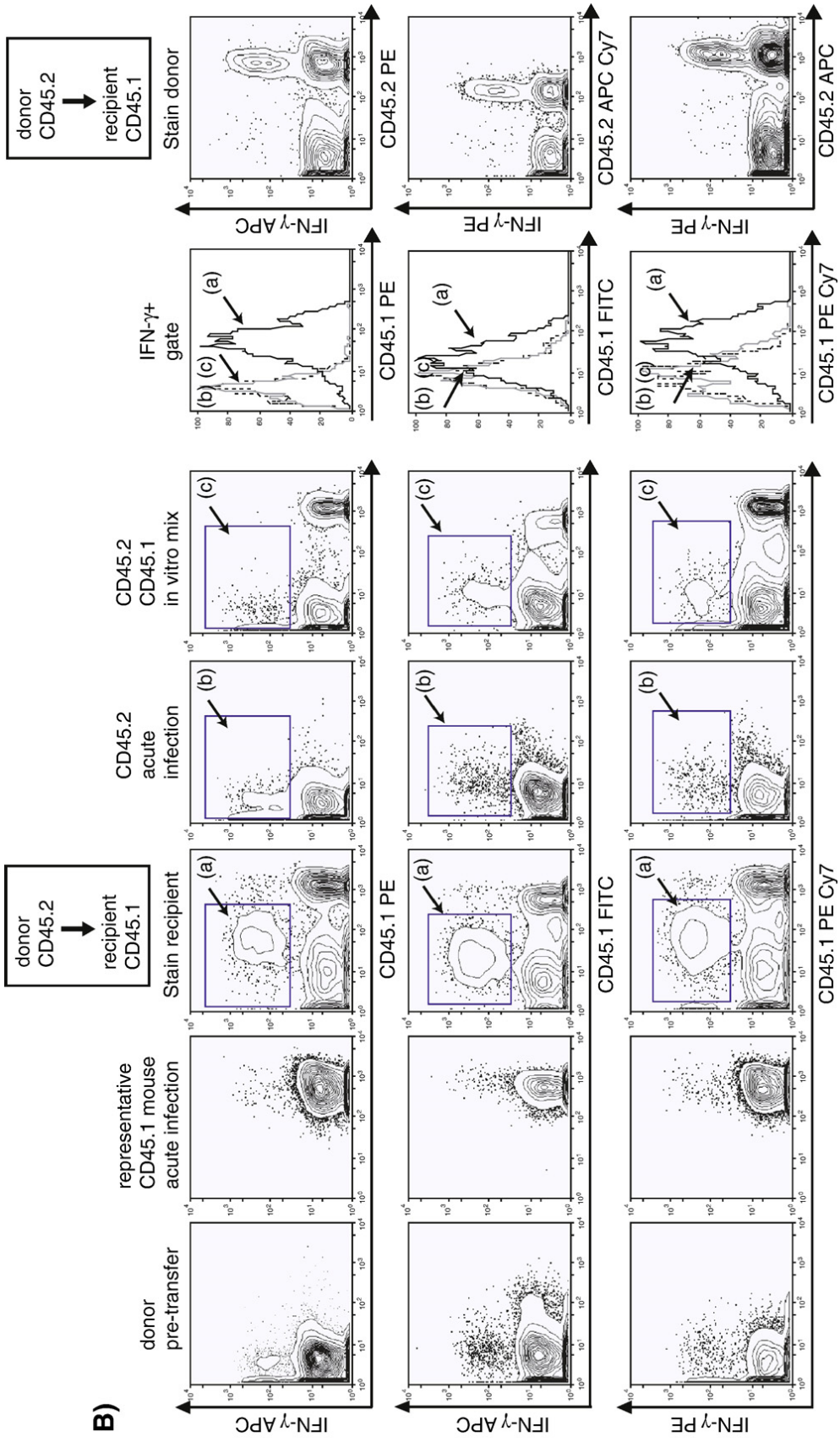

Fig. 2 (*continued*).

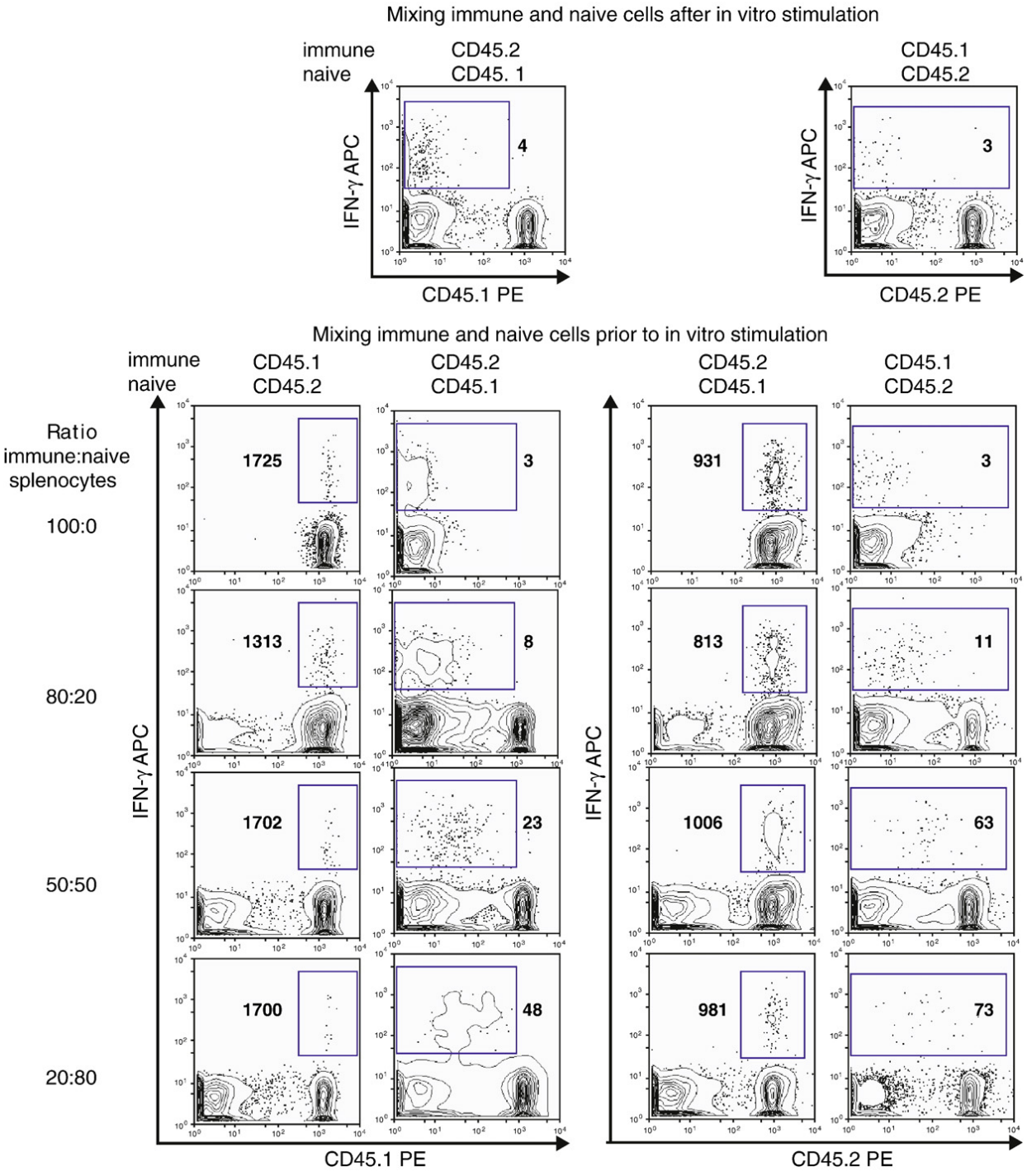

Fig. 3. Membrane transfer from naïve splenocytes to immune CD8 T cells in vitro during ICS incubation. Splenocytes from immune and naïve mice were mixed at different ratios (total 10^6 cells/well) and stimulated with M38 peptide. IFN-γ production was determined by ICS. Numbers in FACS plots refer to mean fluorescence intensity of IFN-γ^+ cells. Staining for CD45.1 immune (column 1) and naïve (column 2) cells and CD45.2 immune (column 3) and naïve (column 4) cells. For comparison, naïve and immune cells mixed at 50:50 ratio after peptide stimulation, immediately before staining is shown in the top two FACS plots.

with immune, peptide-stimulated cells immediately prior to FACS analysis. It should also be noted that the same shift did not occur when identical antibodies and fluorochrome combinations were used to stain the donor origin cells in the reciprocal transfer experiment: compare the last column in Fig. 2A with the third

column in Fig. 2B. This experiment ruled out the possibility that the CD45 marker phenomenon could be an artifact due to inadequate compensation, or due to differences between CD45.1 and CD45.2 behavior or antibodies. Instead, the results suggested that donor origin cells had come to express some of the recipient's congenic marker.

There are many reports that activated T cells can acquire membrane from APCs upon the dissociation of an immunological synapse (Cone et al., 1972; Huang et al., 1999; Hudrisier et al., 2001; Stinchcombe et al., 2001; Tomaru et al., 2003; Wetzel and Parker, 2006). It seemed likely that this phenomenon could account for the phenomenon that we observed. Since recipient cells vastly outnumber donor cells, antigen-bearing cells are predominantly of recipient phenotype. Hence, activated donor origin immune cells could acquire the recipient CD45 marker upon dissociation from antigen-bearing cells.

To test this possibility further, we performed an experiment in which immune cells of one congenic marker were mixed with naïve cells bearing the other congenic marker in vitro. We reasoned that increasing the naïve:donor ratio would result in a greater acquisition of the naïve congenic marker and a gradual increase in fluorescence on immune cells. Immune and naïve cells were cultured together for 6 h in the presence of BFA and peptide in a standard ICS assay. As expected, IFN-γ^+ cells expressed some of the naïve splenocytes' CD45 congenic marker (Fig. 3). The amount of naïve congenic marker expressed on immune cells increased as the proportion of naïve cells in the culture increased, clearly demonstrated by the steady increase in mean fluorescence intensity. If the cell populations were separately stimulated in vitro and mixed immediately before staining, no marker acquisition occurred. These results recapitulate the phenomenon of membrane transfer described by others, and show that membrane transfer is the likely explanation for the misidentification of recipient origin cells we described in Fig. 1.

In the above experiment and most of the instances described in the literature, transfer of APC membrane to T cells occurred in vitro (Cone et al., 1972; Huang et al., 1999; Hudrisier et al., 2001; Stinchcombe et al., 2001; Tomaru et al., 2003; Wetzel and Parker, 2006). However, membrane transfer in vivo has also been described (Walker and Mannie, 2002; Kennedy et al., 2005), and we wondered whether in vivo membrane transfer could also complicate interpretation of adoptive transfer experiments. To further explore this possibility, we performed an adoptive transfer experiment similar to those described above, transferring immune CD45.2 cells into a naïve CD45.1 recipient followed by MCMV infection. However, in this experiment, we used an MHCI tetramer (APC-conjugated) to identify antigen-specific populations, and co-stained with anti-CD45.1 FITC and anti-CD45.2 PE antibodies (Fig. 4). As a control, a CD45.2 congenic mouse was acutely infected with MCMV (Fig. 4A). In an animal that had received cells, the total CD8 T cell population could be separated into three populations (Fig. 4B). The majority of cells were of the recipient phenotype (designated as (d), CD45.1$^+$, CD45.2$^-$), whereas around 10% displayed donor phenotype (designated as (b), CD45.1$^-$, CD45.2$^+$). About 0.6% of CD8 T cells stained positive for both CD45.1 and CD45.2 (designated as (c)). We next stained cells at 4 °C with MHCI tetramer to identify antigen-specific CD8 T cells. Fig. 4C shows the results for the M38 epitope, which elicited the largest response in this experiment. Approximately 76% of tetramer positive cells were CD45.1$^-$, CD45.2$^+$, i.e. of donor origin. A significant recipient population (CD45.1$^+$ CD45.2$^-$), about 23%, was also identified. There was also a small, clearly double positive population, which we interpret as donor cells that had acquired CD45.1 from recipient APC membranes in vivo. However, the donor or recipient origin of the majority of tetramer positive cells could clearly be discerned with either CD45.1 or CD45.2 staining, in distinct contrast to the ICS experiments shown in Figs. 1 and 2. Thus, we conclude that although membrane transfer did occur in vivo, this did not cause misidentification of cells in FACS analysis. Instead, the majority of membrane transfer that led to erroneous interpretation occurred in vitro, during the incubation with peptide for the ICS assay.

One additional feature of the staining in Fig. 4 is worth noting. In addition to the clearly double positive population, the level of CD45.1 fluorescence on the "negative" population was higher compared to that of an acutely infected control CD45.2 mouse with no transfer (top histogram). This shift in the mean fluorescence intensity of the negative population was also seen for the IFN-γ negative populations in the previous experiments. Regardless of whether the donor cells were CD45.1 or CD45.2, their mean fluorescence for the recipient marker was increased slightly when transferred into a congenic mouse subsequently challenged with virus. Since the reciprocal shift in fluorescence was not observed (i.e. the mean fluorescence of the donor marker on recipient cells did not alter), this shift does not seem to be due to compensation issues. We tentatively conclude that the majority of transferred CD8

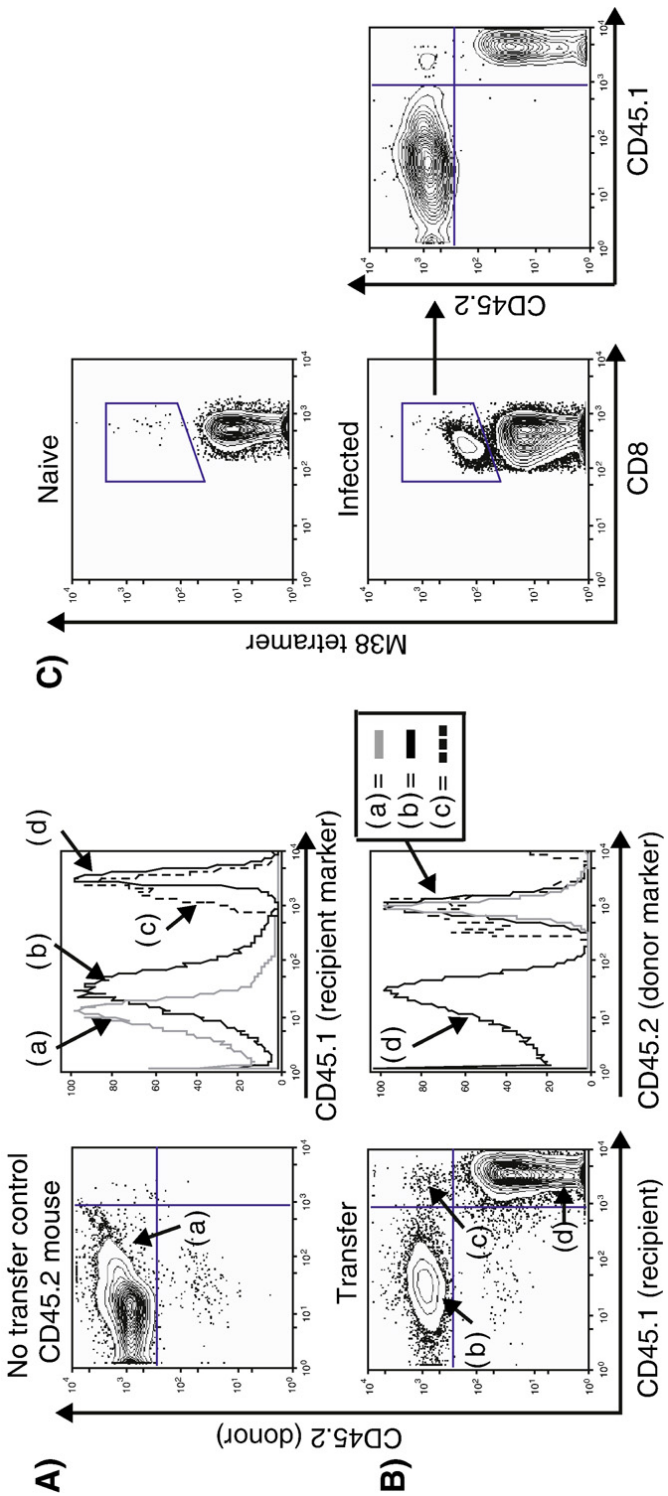

Fig. 4. Evidence of membrane transfer in vivo. Immune splenocytes from a CD45.2 mouse were transferred into a naïve CD45.1 recipient, which was infected with MCMV the next day. One week later, splenocytes were stained for CD8, CD45.1, and CD45.2 and with M38 tetramer. A) CD45.1 and CD45.2 staining on $CD8^+$ splenocytes from an acutely infected CD45.2 mouse. B) CD45.1 and CD45.2 staining on total $CD8^+$ splenocytes from infected CD45.1 mouse that had received CD45.2 immune cells. The histograms show CD45.1 and CD45.2 staining on the indicated gated populations C) CD45.1 and CD45.2 staining of M38 $tetramer^+$ cells.

T cells acquired some recipient membrane during the seven days of acute MCMV infection.

In summary, our experiments show that adoptively transferred immune cells can acquire the recipient CD45 congenic marker, most likely as a result of transfer of APC membrane. This problem was most marked in ICS assays, where CD8 T cells presumably acquired membrane from the peptide pulsed splenocytes in the culture, which were mostly of recipient origin. When donor and recipient cells are distinguished by staining for the recipient's congenic marker, this event can lead to incorrect interpretation of the results, with the transferred cells being misidentified as cells derived from the recipient. Of the two congenic markers most commonly used, CD45 is expressed more widely, including on professional antigen presenting cells and B cells, whereas CD90 (Thy1) expression is mainly limited to T lymphocytes. We did not determine whether Thy 1 staining was also subject to the same problem. However, it seems likely that recipient T cells incubated with peptide during the ICS assay may be recognized by donor CD8 T cells, and could also donate Thy1 bearing membrane to the donor cells.

Membrane transfer is likely to cause problems in interpretation of adoptive transfer assays only in a specific set of circumstances: when using ICS to identify antigen-specific cells and when staining for the recipient's congenic marker. We conclude that, using CD45 as a congenic marker, recipient cells are more accurately identified as the negative population for the donor marker, rather than specifically staining for the recipient's marker. However, staining for both the donor and recipient markers provides the most definitive separation between transferred and recipient cells, and eliminates any uncertainty as to the origin of the cell population.

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