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Research paper

Fluorescent target array T helper assay: A multiplex flow cytometry assay to measure antigen-specific $CD4^+$ T cell-mediated B cell help *in vivo* ☆

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ABSTRACT

$CD4^+$ T cells play a central role in regulating the immune response. Their effector function is commonly assessed by their capacity to secrete cytokines detected by ELISPOT and intracellular cytokine staining. However, one aspect of their effector function that is often overlooked is their ability to help activation of cognate B cells directly, a process that is initiated through the engagement of their T cell-receptor (TCR) with cognate peptide presented on major histocompatibility complex class II (MHC-II) molecules by B cells. Here we report a method to monitor $CD4^+$ T cell-mediated B cell help *in vivo* using a multiplex high throughput assay. This assay utilizes a fluorescent target array (FTA), which is composed of lymphocytes labeled with numerous (>200) unique fluorescence signatures that can be delineated in a single recipient animal based on combination labeling with the three vital dyes carboxyfluorescein diacetate succinimidyl ester (CFSE), CellTrace Violet (CTV) and Cell Proliferation Dye eFluor 670 (CPD). By pulsing different B cell populations in a FTA with titrated amounts of cognate MHC-II binding peptides, $CD4^+$ T cell help could be assessed by measuring induction of the B cell activation markers CD69 and CD44 by antibody labeling and flow cytometry. We call this the “FTA T helper assay”, and have found it to be a robust and sensitive assay to measure $CD4^+$ T cell helper activity across a multitude of peptide-pulsed B “target” cells in real time *in vivo*. Furthermore, the technique can be used simultaneously with the FTA killing assay that measures cytotoxic T cell function, to provide a comprehensive tool for measuring both $CD4^+$ and $CD8^+$ T cell activity during an immune response *in vivo*.

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1. Introduction

$CD4^+$ T cells play a central role in immune regulation, which is underscored by their capacity to differentiate into multiple T cell subsets with unique function (Zhu et al., 2010). T_H1, T_H2 and T_H17 $CD4^+$ effector T cell subsets are characterized by their production of the cytokines IFN-γ, IL-4 and IL-17/22, respectively (Sakaguchi et al., 2008; Fazilleau et al., 2009; Zhu et al., 2010). iT_{REGS} (Sakaguchi et al., 2008) and T_{FH} (Fazilleau et al., 2009) $CD4^+$ effector T cell subsets are notable for their capacity to suppress immune responses (iT_{REGS}) and regulate B cell differentiation (T_{FH}). $CD4^+$ effector T cells are an important indicator in immunotherapy design, but are often overshadowed due to the emphasis placed on generating robust cytotoxic T lymphocyte (CTL) and antibody responses (Rappuoli, 2007). Commonly, $CD4^+$ T cell effector activity is measured by MHC-II/peptide tetramer reactivity (Nepom, 2012) and their capacity to produce cytokines as measured by ELISPOT (Czerkinsky et al., 1983) and intracellular cytokine staining (Prussin and Metcalfe, 1995). However, one aspect of their effector function that is often overlooked is their ability to help activation of cognate B cells directly. $CD4^+$ T helper (T_H) cells modulate B cell differentiation into plasma cells, dictating their fate as extrafollicular short-lived plasma cells or long-lived

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plasma cells and their capacity to switch antibody classes and become high affinity B cells (Fazilleau et al., 2009; Zhu et al., 2010). The fundamental interaction that initiates these events is via the TCR of the effector T_H cell engaging cognate peptide presented on MHC-II by the B cell. As a result of this antigen-specific interaction the B cell is "helped" to undergo activation and differentiation via receptor ligand interactions and paracrine cytokine signaling (Fazilleau et al., 2009; Zhu et al., 2010). Given the importance of this interaction, we sought to investigate whether a method could be developed to monitor T_H cell-mediated B cell help in an *in vivo* setting in a multiplex high throughput assay that could be combined with other T cell effector read outs by flow cytometry.

Recently we utilized the three vital dyes CFSE, CTV and CPD to label lymphocytes with numerous (>200) unique fluorescence signatures based on the different fluorescence emission spectra of the dyes and using multiple fluorescence intensities of the dyes (Quah et al., 2012; Quah et al., 2012). These lymphocyte fluorescent target arrays (FTAs) were used as targets, after pulsing them with MHC-I binding peptides, in an *in vivo* CTL killing assay (Quah et al., 2012). Since more than 200 targets could be detected by this technique the assay allowed the simultaneous measurement of the *in vivo* killing of many target cell clusters pulsed with numerous peptides at different concentrations and the inclusion of many replicates. To expand on this technique, here we report the use of the FTA to measure the capacity of T_H cells to activate B cells within an FTA pulsed with cognate MHC-II binding peptides through measuring upregulation of the B cell activation markers CD69 and CD44. We found the assay to be highly sensitive, detecting antigen-specific, peptide dose-dependent upregulation of activation markers on B cells induced by scarce T_H cells *in vivo*. The FTA T helper assay generates reproducible results that correlate well with the number of antigen-specific $CD4^+$ T effector cells generated *in vivo*. This assay can be coupled with the FTA killing assay to allow the simultaneous measurement of T_H cell and CTL effector activity against a multitude of target cells pulsed with a broad concentration range of several different MHC binding peptides in real time *in vivo*, making it a valuable screening tool for assessment of immune responses.

2. Methods

2.1. Animals

Mice were obtained from the Australian National University (ANU) Bioscience Services, ANU. Mice were housed and handled according to the guidelines of the ANU Animal Experimentation Ethics Committee. Mouse strains used were C57BL/6 (B6), B6.CD45.1 (B6 congenic for CD45.1) and BALB/c. Transgenic (Tg) mouse strains were OT-II (TCR-Tg specific for ISQAVHAAHAEINEAGR (ISQA)/IA^b on a B6 ($CD45.1^+/2^+$) background (Barnden et al., 1998)), OT-I (TCR-Tg specific for SIINFEKL/K^b on a B6 background (Hogquist et al., 1994)). Male mice were used at 6–10 weeks of age.

2.2. Cell preparation

Lymphocytes were obtained from spleen and $CD4^+$ T cells and $CD8^+$ T cells enriched from lymphocytes by MACS separation (Miltenyi Biotec) as previously described (Quah et al., 2004).

2.3. Peptides and virus

Peptides were synthesized at the Australian Cancer Research Foundation Biomolecular Resource Facility, JCSMR, ANU. Peptides included the MHC-II-binding peptides ISQAVH AAHAEINEAGR (ISQA), which is recognized by the OT-II Tg TCR in B6 (IA^b) mice, and PVGEIYKRWIILGLN (Gag Th, a HIV gag epitope recognized by $CD4^+$ T cells in BALB/c ($H\text{-}2^d$) mice (Mata and Paterson, 1999)) and the MHC-I-binding peptides SIINFEKL (which is recognized by the OT-I Tg TCR in B6 (K^b) mice), ASNENMDAM (NP68, an in Flu NP epitope recognized by $CD8^+$ T cells in B6 (D^b) mice), SPYAAGYDL (F2L, a Vaccinia virus epitope recognized by $CD8^+$ T cells in BALB/c (L^d) mice), SPGAAGYDL (F2L mod, a Modified Vaccinia Ankara Virus epitope recognized by $CD8^+$ T cells in BALB/c (L^d) mice), AMQMLKETI (HIV gag, a HIV gag epitope recognized by $CD8^+$ T cells in BALB/c (K^d) mice), AMQMLKDTI (HIV gag mut, a HIV gag subtype C variant of HIV gag (Earl et al., 2009)), VGPTPVNII (HIV pol, a HIV pol epitope recognized by $CD8^+$ T cells in BALB/c (D^d) mice), and RGPGRAFVTI (HIV envelop (env), a HIV env epitope recognized by $CD8^+$ T cells in BALB/c (D^d) mice (Takeshita et al., 1995)). Fowl pox virus (FPV)-HIV and Vaccinia virus (VV)-HIV (VV-HIV (Gag/Pol) and VV-HIV (Env)) stocks were prepared as described previously (Ranasinghe et al., 2006). The VV-OVA construct (Restifo et al., 1995) was kindly provided by Dr D. C. Tscharke.

2.4. Fluorescent dye labeling

CFSE, CTV and CPD dye labeling was as described previously with slight modification (Quah and Parish, 2012). Briefly, splenocytes in 20 °C RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (FCS), were labeled with 0–66,000 nM of each dye in 1–2 mL aliquots for 5 min and then washed ≥3 times (Quah and Parish, 2010, 2012). The membrane intercalating dye DiI (Invitrogen) was used at 14 μM to label cells as described by the manufacturer.

2.5. FTA preparation

FTA preparation was as described previously (Quah et al., 2012). Briefly, 2 mL aliquots of splenocytes were initially labeled with several concentrations (dependent on the type of FTA) of CTV. Cells were then split equally and labeled with several concentrations of CFSE and then washed once. Cell aliquots were then incubated with MHC-I and MHC-II binding peptides for 1 h at 37 °C, and washed twice at 4 °C, with the first wash being through a FCS cushion. Samples to be labeled with the same concentration of CPD were then pooled and washed once more, before being labeled with various concentrations of CPD. After washing the cell samples twice, all aliquots were pooled and washed again. Where necessary, pooled cells were also labeled with DiI. The FTA was then counted and resuspended at up to 25×10^7 cells/mL ready for injection.

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