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The activation, by antigen, of naïve TCR transgenic CD4 T cells cultured at physiological, rather than artificially high, frequencies more accurately reflects the *in vivo* activation of normal numbers of naïve CD4$^+$ T cells

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ABSTRACT

The majority of *in vitro* studies investigating the activation of naïve TCR transgenic T cells routinely employ an artificially high frequency of such cells. To assess whether employing high frequencies of TCR transgenic cells *in vitro* accurately reflects the *in vivo* activation of a normal number of T cells, we cultured between 300 and 3×10^6 Rag2$^{-/-}$ DO11.10 T cells per well under otherwise identical conditions. We find that those T cells cultured at low frequencies proliferate more and are more potently activated, as assessed by the expression of CD44 and CD62L, each giving rise to a much larger number of cytokine producing cells, comparable to the number generated *in vivo* when a normal number of CD4$^+$ T cells are activated. The effect of T cell frequency on the level of their activation was not due to differences in MHCII or CD80/86 expression by B cells, the major APC population present, nor to increased death of B cells in high frequency cultures. Taken together, our observations illustrate the necessity of culturing naïve TCR transgenic CD4$^+$ T cells at a physiological frequency if one is to more accurately recapitulate the *in vivo* activation of naïve CD4$^+$ T cells.

1. Introduction

In a mouse, the number of precursor CD8$^+$ and CD4$^+$ T cells present for certain pathogens, has been estimated to be on average 200 per immunogenic epitope [1–5] or approximately 1000–14,000 for an entire pathogen [6,7]. Several groups have found that adoptively transferring a number of TCR transgenic T cells, beyond the number normally present for an antigen, significantly affects their activation. For example, when the activation of artificially large numbers of TCR transgenic cells is compared with the activation of normal polyclonal T cells or fewer transgenic cells, it is found that there is less proliferation [8,9], a smaller yield of cytokine producing cells [8,9], less susceptibility to co-stimulation blockade [8], and poorer survival [10]. Additionally, the adoptive transfer of large numbers of TCR transgenic T cells, prior to immunization of the animal with the appropriate antigen, has been shown to alter the commitment of T cells to a memory phenotype [3,11]. Clearly, the responses obtained from large numbers of TCR transgenic T cells *in vivo* do not reflect responses generated by T cells present at a normal physiological frequency.

A high frequency of TCR transgenic T cells is routinely employed *in vitro* to investigate various aspects of naïve T cell activation. In many cases the utilization of a high frequency of T cells is necessary to realize sufficient numbers for analysis; this is particularly true for quantifying cytokines by ELISA, which requires a large, and therefore detectable, amount of cytokine in the culture supernatant. An alternative to ELISA for determining the cytokine profile of activated T cells is the ELISpot assay. The ELISpot is a very sensitive assay that detects cytokine producing cells. In contrast to traditional ELISpot assays, our published protocol involves the addition of naïve splenocytes to ensure that the number of APC is non-limiting [12]. This modification both increases the sensitivity of the assay and allows its use to enumerate antigen-specific cytokine producing cells present at very low numbers. Employing our modified ELISpot assay, we report herein that we are able to routinely enumerate the number of cytokine producing cells present in wells that initially contained as few as 300 naïve TCR transgenic T cells.

In light of the above mentioned *in vivo* observations, we wished to investigate whether the use of a high frequency of TCR transgenic T cells *in vitro* accurately reflects the activation of a normal frequency of T cells *in vivo*. In order to address this question we assessed the activation, by ELISpot and by flow cytometry, of 300–3×10^6 Rag2$^{-/-}$ DO11.10 T cells, which express a transgenic TCR that recognizes the OVA$_{323-339}$ peptide in the context of I-A^d, cultured under identical conditions.

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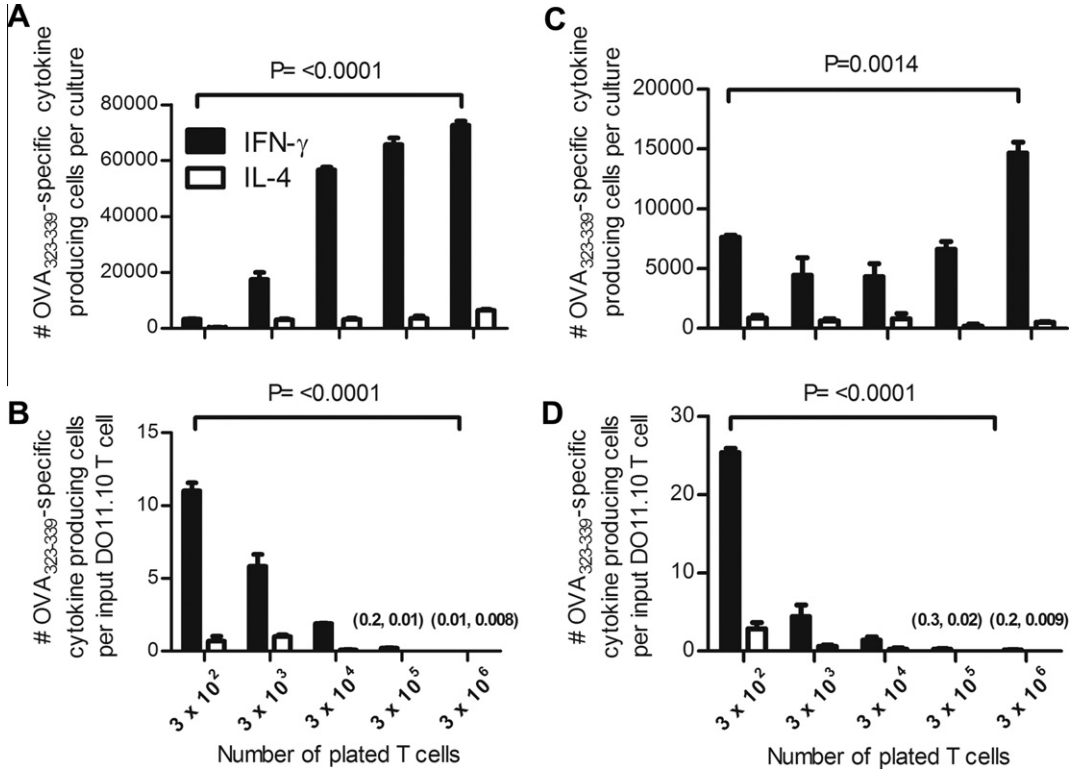

Fig. 1. The frequency of responding CD4$^+$ T cells affects the number of cytokine producing cells derived from a single precursor *in vitro*. The indicated number of purified CD4$^+$ Rag2$^{-/-}$ DO11.10 T cells were cultured with 3×10^6 T cell depleted BALB/c splenocytes and either 0.3 μM $OVA_{323-339}$ peptide (A and B) or 3 μM OVA protein (C and D in a single well of a 24-well tray). On day 4 (A and B) or day 7 (C and D), the cultures were harvested and the $OVA_{323-339}$-specific IFN-γ and IL-4 secreting cells per well were enumerated by ELISpot assay. The data in panels A and C were normalized to the initial number of plated CD4$^+$ Rag2$^{-/-}$ DO11.10 T cells and were plotted in panels B and D, respectively. The numbers in parentheses indicate, in order, the number of IFN-γ and IL-4 producing cells. Error bars represent the standard deviation of the mean of three replicate wells. Statistical significance was assessed using a *T*-test. This figure is representative of three independent experiments.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from Charles River, Canada. Rag$^{-/-}$ DO11.10 mice (C.Cg-Rag2^{tm1Fwa} Tg (DO11.10) 10Dlo) were obtained from Taconic Farms. All experiments were approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2. Preparation of T cells and APC

Splenic DO11.10 CD4$^+$ T cells were purified by negative selection with the CD4$^+$ T cell isolation kit II (Miltenyi) as per the manufacturer's instructions. The APC used throughout the study were BALB/c splenocytes depleted of T cells by employing CD90.2 microbeads (Miltenyi) as per the manufacturer's instructions.

2.3. In vitro culture

The indicated number of CD4$^+$ T cells were plated with the indicated number of T cell depleted BALB/c splenocytes and antigen in a single well of either a 96-well V-bottom tray (Corning) or a 24-well flat bottom tray (BD biosciences). The media used for culture was RPMI supplemented with 10% heat-inactivated fetal calf serum (Hyclone), 100 U/mL penicillin–streptomycin (Gibco), 0.8% sodium pyruvate and 50 μM β-mercaptoethanol. The antigen used in culture was either 3 μM ovalbumin protein (Sigma) or 0.3 μM ovalbumin peptide amino acid residues 323–339 (Genscript; $OVA_{323-339}$). Where indicated, functional grade anti-CD28 (37.51, ebioscience) or functional grade isotype control antibody were added to the cultures at a final concentration of 30 μg/mL.

2.4. ELISpot

$OVA_{323-339}$-specific cytokine producing cells were enumerated using an optimized ELISpot assay as previously described [12]. Briefly, the cultures were harvested on the indicated day and washed with RPMI media. The cells were then diluted in RPMI media supplemented with 100 U/mL penicillin–streptomycin (Gibco), 0.8% sterile sodium pyruvate and 50 μM β-mercaptoethanol. Additional naïve BALB/c splenocytes were added to each well to bring the total number of lymphocytes to 1×10^6 per well; this ensures the optimal detection of cytokine producing cells. Peptide-specific cytokine spots were enumerated by subtracting the spots produced in wells without antigen from those produced in the presence of 0.3 μM $OVA_{323-339}$ peptide. The number of spots generated in the presence of antigen was generally more than 50-fold greater than in the absence of added antigen. The total number of cytokine producing cells per culture was calculated by multiplying the number of $OVA_{323-339}$-specific cytokine producing cells by the dilution factor. Where indicated, the number of cytokine secreting cells was normalized to the number of plated DO11.10 T cells by dividing the number of $OVA_{323-339}$ specific spots per culture by the initial number of DO11.10 T cells placed in culture.

2.5. CFSE labeling

1×10^7 freshly isolated CD4$^+$ DO11.10 T cells were labeled with a final concentration of 5 μM CFSE in 1 mL PBS containing 5% FCS for 5 min at room temperature. The cells were then washed three