An *in vitro* method for the identification of DNP- and TNP-specific T cells in the naive compartment of human blood

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Introduction

In the past commonly used methods for the identification of sensitizers involved animal testing. This approach had to be urgently replaced by *in vitro* methods for ethical and legislatory reasons. We developed a new method for the identification of human naive T cells with a certain antigen specificity, including chemical-specific T cells. We used DNP and TNP as models for sensitizing chemicals. The method is based on an *in vitro* T cell culture system to prime naive and expand antigen-specific T cells, followed by the flow cytometric detection of cytokine-producing CD4$^+$ and CD8$^+$ T cells.

Material and methods

Monocytes were isolated from PBMC of healthy donors using CD14 MicroBeads and the autoMACS® Pro Separator. An aliquot of purified monocytes was cryopreserved for later use. The CD14$^-$ cell fraction was used to magnetically enrich naive T cells by depletion of non-T cells, CD45RO$^+$ cells, and CD25$^+$ cells. T cells were either cultured for 2 days or cryopreserved until DCs were differentiated. Feeder cells were generated by treatment of the T cell–depleted cell fraction with mitomycin C. Monocytes were differentiated into DCs in the presence of IL-4 and GM-CSF. DCs were subsequently modified by resuspending cells in 1 mL of a 4 mM DNBS or 3 mM TNBS stock solution. The T cell priming culture was set up using 10^7 T cells, 10^6 DCs, and 5×10^6 feeder cells and CD28 pure antibody. IL-7 (10 ng/mL) and IL-15 (10 ng/mL) were added on days 2 and 6, and IL-2 (100 IU/mL) on days 4 and 6. All cultures were maintained using RPMI 1640 + 5% AB-serum.

T cells were harvested on day 8, washed with cytokine-free medium, rested for 1 day, and restimulated with modified DCs, which were generated from cryopreserved monocytes. Cytokine expression by T cells was analyzed by flow cytometry after intracellular staining.

Results

1 Identification of DNP- and TNP-specific T cells

DNP- and TNP-specific T cells were detectable in all samples tested from three donors for each specificity, using the protocol described in figure 1A. Antigen specificity of *in vitro* primed and expanded T cells as well as the specificity of the detection method were evaluated by restimulating TNP-primed T cells with DNP-modified MoDCs and DNP-primed T cells with TNP-modified MoDCs (fig. 1B). Only a limited frequency of T cells within the TNP- or DNP-primed cultures responded to DNP- or TNP-modified MoDCs, respectively. This confirms the validity of the assay and the antigen-specificity of the T cells. The small frequency of T cells reacting to TNP- as well as DNP-modified MoDCs may be explained by cross-reactivity due to the high similarity of the TNP and DNP structures.[1]

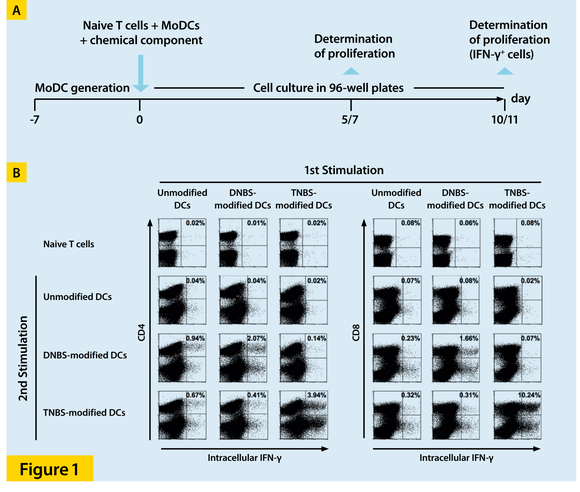


Figure 1

2 FastDCs as APC for priming and restimulation of chemical-specific T cells

To avoid unnecessarily long culture periods we examined the capability of monocytes differentiated for only 2 days (fastDCs) instead of 7 days (MoDCs) for antigen processing, presentation, and T cell activation in a) restimulation cultures (fig. 2A) and b) priming cultures (fig. 2B). Short-term restimulation of either PBMC or of antigen-experienced T cells from CMV-infected donors with soluble CMV IE-1 protein or pulsed fastDCs or MoDCs showed that both fastDCs and MoDCs were able to effectively activate IE-1-specific CD8$^+$ T cells, whereas monocytes did not induce T cell activation. This result indicates that both MoDCs and fastDCs cross-present exogenously delivered protein antigens into the MHC-class-I pathway, leading to the activation of CD8$^+$ T cells.

Hence, we replaced TNP-modified MoDCs with TNP-modified fastDCs for priming of naive T cells and restimulation, which allowed us to identify TNP-specific CD4$^+$ and CD8$^+$ T cells, establishing fastDCs as competent APCs. Moreover, due to the shorter culture period, the viability and recovery of fastDCs is increased compared to MoDCs and cryopreservation of T cells prior to the priming culture is not required.

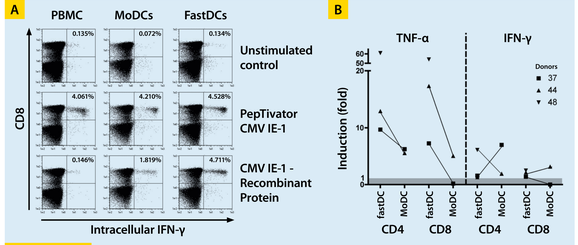
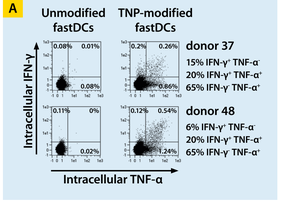


Figure 2

3 Intracellular IFN-γ and TNF-α staining as a readout system for chemical-specific T cells

Since not all chemicals may induce IFN-γ production, we included the analysis of other effector cytokines (TNF-α, IL-17, and IL-22). So far we did not find IL-17 or IL-22 expression in TNP-primed and restimulated cell cultures, but TNF-α was clearly detectable in all tested samples. Co-expression analysis of TNF-α and IFN-γ revealed that cells within the TNP-reactive T cell population produce either TNF-α or IFN-γ, or both cytokines (fig. 3A).



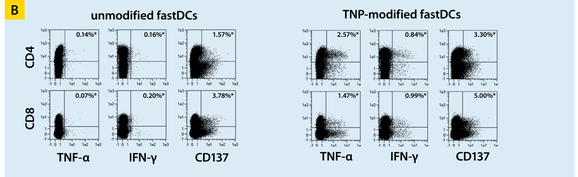


Figure 3 * Frequency among CD4$^+$ or CD8$^+$ T cells

Furthermore, we examined the suitability of the activation marker CD137 as a readout, as this may allow the identification of antigen-specific cells irrespective of the expression of effector cytokines. However, CD137 expression was in part independent of the restimulation with or without antigen, excluding CD137 as a marker for the identification of antigen-specific T cells (fig. 3B). We therefore recommend the combined detection of IFN-γ and TNF-α by intracellular cytokine staining as readout system.

4 Cytokines for T cell priming and expansion culture

Next we re-examined whether addition of the recombinant cytokines IL-2, IL-7, and IL-15 during T cell priming and expansion is required for optimal expansion and survival of chemical-specific T cells. We found that exogenous cytokines are essential for our test system (fig. 4) since culturing cells without the cytokines led to massive cell death, and chemical-specific T cells were not detectable.

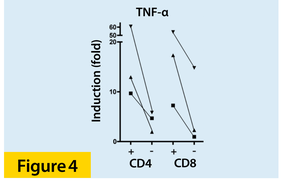


Figure 4

Summary

In summary, we established a fast and robust *in vitro* assay to identify contact sensitizer–primed T cells (fig. 5). There are still some issues to be solved for the development of an assay that allows a prediction of the allergenicity of a particular chemical: the chemicals' cell toxicity and the way of delivery to the assay. Using chemical-modified human serum albumin as antigen might be a suitable approach to discriminate sensitizers and non-sensitizers in this T cell priming assay.

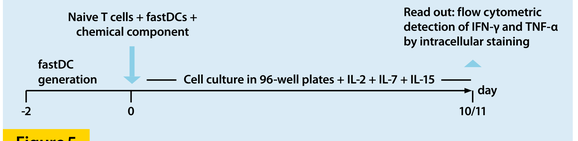


Figure 5

Reference
1. Dietz, L. *et al.* (2010) Toxicol. Sci. 117: 336–347.