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Research paper

Large scale expansion of Vγ9Vδ2 T lymphocytes from human peripheral blood mononuclear cells after a positive selection using MACS "TCR γ/δ + T cell isolation kit"

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ABSTRACT

Interest in γ9δ2 T cells has increased greatly in the past decade. While several protocols allowed the amplification of a large proportion of these cells *in vitro*, the purity of the final preparation is usually heterogeneous between different donors. Functional studies of this population are often controversial due to the presence of other populations such as NK cells which share a wide range of characteristics. Here, the γ9δ2 T cells labelled-fraction is purified and mixed with the irradiated unlabelled fraction followed by a single stimulation with phosphoantigen, in turn followed by a classical step of amplification in the presence of interleukin 2. In this study, we describe a straightforward protocol to amplify pure populations of γ9δ2 T cells which could be useful in fundamental research or in the development of a new generation of γδ cell therapy protocol.

1. Introduction

The ability of γδ T lymphocytes to respond to non-processed and non-peptidic phosphoantigens in an MHC-unrestricted manner distinguishes γδ T lymphocytes from αβ T lymphocytes (Brenner et al., 1987; Constant et al., 1994; Bukowski et al., 1995; Tanaka et al., 1995). γδ T lymphocytes regulate the initiation, progression and resolution of the immune response to infectious pathogens and γδ T-cell-mediated immune responses have been demonstrated in many microbial infections, suggesting that anti-viral immuno-surveillance may be one of the primary γδ T-cell functions (Poupot and Fournie, 2004). More than 70% of the circulating human γδ T cells express the Vγ9Vδ2 TCR chains (Parker et al., 1990), representing 0.5–5.0% of whole peripheral blood T cells. Besides their anti-infectious activity, γ9δ2 T lymphocytes participate, together with dendritic cells (DC) (Kroca et al., 2001), NK cells, and NK-T cells, in the innate immunity response to cancer processes (Walzer et al., 2005). Many cytotoxicity assay studies have been shown that γ9δ2 T lymphocytes are able to kill a wide variety of tumour cell lines from very diverse origins (Sturm et al., 1990; Fisch et al., 1997; Burjanadzé et al., 2007; Bank et al., 1993; Fujimiya et al., 1997; Mitropoulos et al., 1994; Zheng et al., 2001; Otto et al., 2005; Corvaisier et al., 2005; Ferrarini et al., 1996; Bouet-Toussaint et al., 2008).The first clinical evidence for *in vivo* manipulation of human γ9δ2 T lymphocytes induced by γ9δ2 T-cell agonists came unexpectedly from the finding of circulating γ9δ2 T lymphocyte increases in some adults with multiple myeloma (MM) following intravenous injection of the aminobisphosphonate pamidronate. Moreover, this population could also be expanded *in vitro* with phosphoantigen and used for cell therapies clinical approaches (Lopez et al., 2000; Bennouna et al., 2008; Kobayashi et al., 2001). Despite these encouraging results, *in vitro* studies of this population after a single

Abbreviations: BrHPP, Bromohydrin Pyrophosphate;FCS, Fetal Calf Serum; MNC, MonoNuclear Cell;TNFα, Tumour Necrosis Factor α.

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stimulation by phosphoantigen (such as BrHPP or C-HDMAPP) are often controversial due to the presence of other populations which share a range of characteristics in the final preparation. Therefore, we developed a straightforward protocol to amplify pure populations of γ9δ2 T cells which could be of use in fundamental research or in the development of a new generation of γδ cell therapy protocol.

2. Time required

The first step (Day 0) takes approximately 3 h 30:

- Isolation of PBMC: 2 h.
- Selection of TCR γ/δ^+ cells with Anti-TCR γ/δ Microbead Kit (Miltenyi, ref: 130-050-701): 1 h.
- Culture initiation: 30 min.

The second step depends on cell culture maintenance needs at each step:

- At day 4: 1 h
- At day 7: 2 h
- At day 10: 1 h

3. Materials

- Human PBMC obtained from a donation of concentrated platelet by apheresis (1 billion cells).
- Ficoll (MSL, Eurobio, France).
- RPMI 1640 with L-glutamine (Lonza Verviers, Belgium).
- Fetal Calf Serum (FCS) (Hyclone, US).
- LS separation columns (Miltenyi Biotec, United States).
- A buffer containing 1 volume of MACS BSA Stock Solution for 20 volumes MACS Rinsing Solution (Miltenyi Biotec, United States).
- TCR γ/δ Microbead Kit (Miltenyi Biotec, United States).
- Interleukin 2 (Novartis, Switzerland).
- BrHPP (PhosphostimÔ, IPH1101, Innate Pharma, France).
- C-HDMAPP (PicostimÔ, IPH1201, Innate Pharma, France).
- 50 cm^3, 5 cm^3 syringes (Terumo, Belgium).
- 25 g syringes needles (Becton Dickinson, United States).
- 600 ml transfer bag (Baxter, United States).
- Life Cell Culture Bag (Miltenyi Biotec, United States).

4. Detailed procedure

4.1. Sample preparation

Human PBMC obtained from donated concentrated platelets by apheresis were separated on a ficoll gradient and washed by centrifugation. One billion cells (containing at maximum 5% of target cells) were used with the TCR γ/δ Microbead Kit (Miltenyi Biotec). The procedure was performed as follows:

- Centrifuge total cells at 300 ×g, 4 °C for 10 min.
- Discard the supernatant.
- Resuspend cell pellet to obtain $10 \cdot 10^6$ total cells in 40 µl of buffer.
- Add 10 µl of Anti-TCR γ/δ Hapten-Antibody at $10 \cdot 10^6$ cells.
- Mix and incubate 10 min at 4–8 °C.
- Add 30 µl of buffer and 20 µl of MACS anti-Hapten MicroBeads at $10 \cdot 10^6$ total cells.
- Mix and incubate for 15 min at 4–8 °C.
- Wash cells by adding 1–2 ml buffer per $10 \cdot 10^6$ total cells.
- Centrifuge at 300 ×g, 4 °C for 10 min.
- Discard the supernatant.
- Resuspend the preparation up to $100 \cdot 10^6$ cells in 500 µl of buffer.

4.2. Magnetic separation

- Place column in the magnetic field of MACS Separator and rinse with 3 ml of buffer.
- Apply cell suspension inside the column.
- Collect unlabeled cells which pass through the column and performed three washing with 3 ml of buffer.
- Remove column from the separator and place it on a collection tube.
- Add 5 ml of buffer in the column.
- Immediately flush out fraction with magnetically labelled cells in using the plunger supplied.
- Place a new column in the magnetic field of MACS Separator and rinse with 3 ml of buffer.
- Add the labelled cell fraction inside this new column.
- Collect cells which pass through and wash column three times with 3 ml of buffer.
- Remove column from the separator and place it in a collection tube.
- Add 5 ml of buffer in the column.
- Immediately flush out fraction with magnetically labelled cells in using the plunger supplied.

4.3. Culture initiation

- Adjust volume of unlabeled cell fraction to 50 ml.
- Count using eosin exclusion.
- Irradiate the unlabeled cell fraction at 35 Gy.
- Count unlabeled cells using eosin exclusion.
- Centrifuge the unlabeled and labelled cell fractions at 400 ×g, 20 °C for 8 min.
- Discard the supernatant.
- Resuspend and mix the unlabeled and labelled cell fractions at $1.5–2.5 \times 10^6$ cells/ml under 500 ml of RPMI 1640-FCS 9% with L-glutamine, IL2 at 60 ng/ml and 3 µM BrHPP.
- Inoculate the mixed preparation in 3-l culture bag.
- Incubate cell suspension at 37 °C, 5% CO_2 for 4 days.

4.4. Culture maintenance

γ9δ2 cells T lymphocytes are expanded for 10 days in a closed system in culture medium with interleukin-2 (IL-2) as previously described (Salot et al., 2007). Cell counts were determined using a Malassez chamber in which cells were diluted with 0.15% eosin in order to evaluate cell viability. The cell amplification factor was calculated as (viable cell number at day 10/viable cell number at day 0). Cell culture maintenance medium was prepared extemporaneously (RPMI-FCS 9% and Il2 60 ng/ml) and prior to addition of the medium, cell suspensions were divided into two when the final volume was upper than 2.2 l. At day 10, in order to determine the cell amplification factor, a cell count was performed.