Video Article

Murine Model of CD40-activation of B cells

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Abstract

Research on B cells has shown that CD40 activation improves their antigen presentation capacity. When stimulated with interleukin-4 and CD40 ligand (CD40L), human B cells can be expanded without difficulties from small amounts of peripheral blood within 14 days to very large amounts of highly-pure CD40-B cells (>10^9 cells per patient) from healthy donors as well as cancer patients[1-4]. CD40-B cells express important lymph node homing molecules and can attract T cells *in vitro*[5]. Furthermore they efficiently take up, process and present antigens to T cells[6,7]. CD40-B cells were shown to not only prime naíve, but also expand memory T cells[8,9]. Therefore CD40-activated B cells (CD40-B cells) have been studied as an alternative source of immuno-stimulatory antigen-presenting cells (APC) for cell-based immunotherapy1,5,10. In order to further study whether CD40-B cells induce effective T cell responses in vivo and to study the underlying mechanism we established a cell culture system for the generation of murine CD40-activated B cells. Using splenocytes or purified B cells from C57BL/6 mice for CD40-activation, optimal conditions were identified as follows: Starting from splenocytes of C57BL/6 mice (haplotype H-2b) lymphocytes are purified by density gradient centrifugation and co-cultured with HeLa cells expressing recombinant murine CD40 ligand (tmuCD40L HeLa)[11]. Cells are recultured every 3-4 days and key components such as CD40L, interleukin-4, -Mercaptoethanol and cyclosporin A are replenished. In this protocol we demonstrate how to obtain fully activated murine CD40-B cells (mCD40B) with similar APC-phenotype to human CD40-B cells (Fig 1a,b). CD40-stimulation leads to a rapid outgrowth and expansion of highly pure (>90%) CD19+ B cells within 14 days of cell culture (Fig 1c,d). To avoid contamination with non-transfected cells, expression of the murine CD40 ligand on the transfectants has to be controlled regularly (Fig 2). Murine CD40-activated B cells can be used to study B-cell activation and differentiation as well as to investigate their potential to function as APC in vitro and in vivo. Moreover, they represent a promising tool for establishing therapeutic or preventive vaccination against tumors and will help to answer questions regarding safety and immunogenicity of this approach[12].

Video Link

The video component of this article can be found at https://www.jove.com/video/1734/

Protocol

The protocol for the generation of murine CD40-activated B cells (mCD40B) from splenocytes is divided into two parts: Part A demonstrates the preparation of murine CD40 ligand (CD40L) expressing HeLa cells (tmuCD40L HeLa), which will be used as plate-bound feeder cells. Part B describes the actual murine CD40-B culture.

A. Preparation of feeder cells (tmuCD40L HeLa)

The tmuCD40L HeLa is an adherent human epithelial cell line, which should never become completely confluent. The cells are therefore splitted twice per week. Culturing over more than 6 weeks is not recommended.

1. Remove old medium from the primary culture with a sterile pipette and wash cells with 10 mL of 1x PBS. Aspirate the PBS after washing.
2. Add 4 mL of 1x Trypsin/EDTA in a 75 cm^2 flask for 10-15 minutes at 37°C. Use gentle tapping to detach the cells.
3. Add 10 mL of wild type medium and swivel gently to stop digestion with trypsin.
4. Transfer the cell suspension into a 50 mL tube with a sterile pipette and spin the cells down at 265 x g for 7 minutes.
5. Remove the supernatant and resuspend the cell pellet in 10 mL of wild type medium. Count the cell number of an aliquot of the cell suspension and prepare three 50 mL tubes with the appropriate number of cells:
    1. 2.5 x 10^6 cells for subculture
    2. 0.4 x 10^6 cells/well for irradiation used for the murine CD40-B cell culture
    3. remainder to freeze (if needed).
6. Spin the cells down at 265 x g for 7 minutes.
7. Remove the supernatant.

1. For subculturing: resuspend 2.5 x 10^6 cells in 10 mL selection medium in a 75 cm^2 cell culture flask (cell density 2.5 x 10^5 cells/mL) and incubate the cells at 37°C with 5 % CO_2. Split the cells twice per week.
2. For the murine CD40-B cell culture: You need 2.4 x 10^6 cells for one 6-well plate. Resuspend the tmuCD40L HeLa cells in wild type medium at a density of 0.2 x 10^6 cells/mL and irradiate them at 78 Gy. Plate 2 mL of the cell suspension into each well and incubate them at 37°C with 5 % CO_2. Use this prepared plates for B-cell stimulation when tmuCD40L HeLa cells are adherent (at least 4 hours: check adherence using the microscope; do not wait more than 24 h to start B-cell stimulation). (Continue with B.)

B. Murine CD40-B cell culture

I. Preparation of splenocytes for CD40-stimulation (day 0):

Please note: before you continue ascertain that feeder cells are adherent. Always add fresh solutions of murine interleukin-4, β-Mercaptoethanol and cyclosporin A to the growth medium immediately before use.

1. Dissect spleen of C57BL/6 mice (haplotype H-2b) and wash it twice in 10-20 mL DMEM supplemented with Gentamycin [15µg/mL] by transferring it with a 10mL pipette tip from one tube to another. Avoid transferring contaminants (e.g. hair) of the mouse. Mince spleen by passing it through a cell strainer (100µm) and rinse strainer with another 10 mL of medium. Spin down splenocytes at 265 x g for 7 min at room temperature, resuspend them in 7mL muCD40-B cell medium, and separate lymphocytes by Ficoll density centrifugation. To do so layer cells on 5 mL Mouse-Pancoll (preheated at room temperature) in a 15 mL tube and centrifuge at 450 x g for 30 min without brake at room temperature.
2. Draw off most of the upper layer, leaving the lymphocyte layer undisturbed at the interface. Transfer lymphocyte layer to a fresh 50 mL tube, wash once with 30mL DMEM with Gentamycin [15µg/mL] by spinning down at 265 x g for 7 min to remove other cells. Discard the supernatant and resuspend the cells in 10 mL of mCD40-B washing medium. Determine the cell number in an aliquot of the cell suspension. Determine cell number by counting an aliquot of the cell suspension.
3. Spin down the required amount of cells at 265 x g for 7 minutes. For a 6-well plate 5 x 10^6 cells/well are needed, thus 30 x 10^6 cells per plate.
4. Remove the supernatant and resuspend the lymphocytes at 1.25 x 10^6 cells/mL in mCD40-B culture medium freshly supplemented with 1 U/mL of interleukin-4 as growth factor, 100 µM β-Mercaptoethanol and 0.63 µg/mL cyclosporin A to prevent outgrowth of T-cells (Given concentrations refer to one mL culture medium!).
5. Remove the supernatant from the 6-well plate pre-incubated with tmuCD40L HeLa cells.
6. Gently add 4 mL of the lymphocyte suspension (1.25 x 10^6 cells/mL) to each well of the 6-well plate.
7. Incubate the plate at 37°C with 5 % CO_2.
8. On day 3 reculture the cells (Continue with II.1.).

II. Subculture of mCD40B cells (day 3 and then twice a week):

1. Harvest mCD40B cell cluster by vigorously pipetting up and down with a 10 mL pipette and pool them into a 50 mL tube.
2. Spin down at 265 x g for 7 minutes and completely replace the supernatant with mCD40-B washing medium. While counting the cell amount of an aliquot, spin the cells down at 265 x g for 7 minutes. Resuspend mCD40B cells in mCD40-B culture medium at a concentration of 0.75 x 10^6 cells/mL.
3. Add fresh solutions of murine interleukin-4 in a concentration of 1 U/mL, 100 µM β-Mercaptoethanol and 0.63 µg/mL cyclosporin A to the medium.
4. Remove the supernatant from a 6-well plate pre-incubated with irradiated tmuCD40L HeLa cells.
5. Gently add 4 mL of the mCD40B cell suspension (0.75 x 10^6cells/mL) to each well of the 6-well plate.
6. Incubate plates at 37°C with 5 % CO_2.
7. Again subculture the cells every 3-4 days to end up with highly pure murine CD40-activated B cells after a total of 14 days.

C. Trouble-shooting: What if CD40-B cells do not grow?

1. Have you checked for CD40 ligand expression of feeder cells?
2. Are the plate-bound feeder cells used for the stimulation older than 24h?
3. Is there a contamination with mycoplasma?
4. Was the interleukin-4 solution used for supplementation freshly thawed and did it have the appropriate biological activity?
5. Was β-Mercaptoethanol and cyclosporin A added in the correct concentration?

1a)

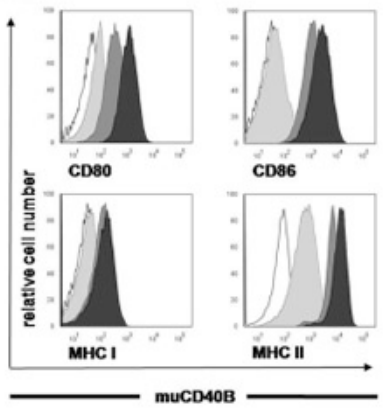


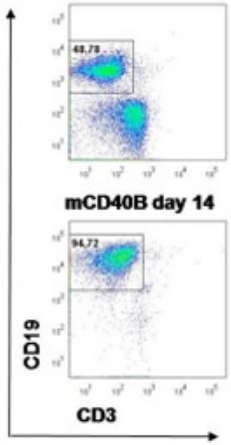
Fig. 1 (a, b). Phenotypical FACS analysis of mCD40B cells:
(a) Phenotype of CD19⁺ mCD40B cells after continuous CD40-stimulation for days 0, 7 and 14 including co-stimulatory (CD80, CD86) and MHC I and II molecules ($I\text{-}A^b$, $H\text{-}2D^b$).
The histogram shaded in light grey, dark grey and black represent days 0, 7 and 14 during CD40-stimulation, respectively. The white histograms represents the isotype control of each antibody.

(b) Determination of the CD19⁺ cell fraction at the beginning and after 14 days of mCD40B culture.

Representative data are shown.

Figure 1a.

b)



c)

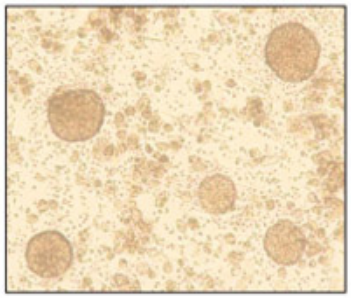


Fig. 1 (c). Clustering of mCD40B cells:
In (c) mCD40B cells under CD40-stimulation for 14 days accumulate to cell cluster due to homotypic aggregation.

Figure 1b.

d)

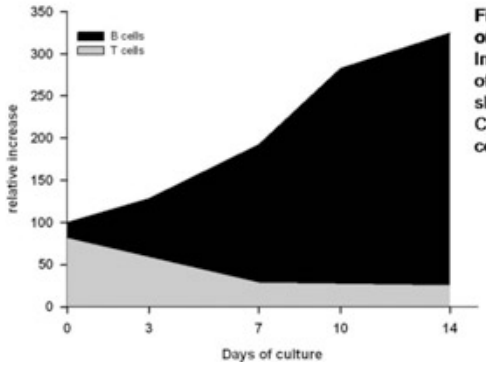


Fig. 1 (d). Quantification of mCD40B cell growth:
In (d) normalized cell count of 5 mCD40B cultures is shown. Values represent CD19⁺ B cells and CD3⁺ T cells.

Figure 1d.

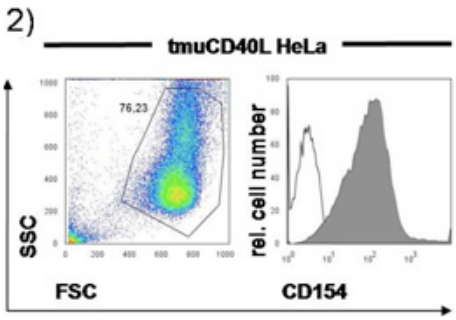


Fig. 2. Phenotypical FACS analysis of tmuCD40L HeLa: Representative data for gating on viable feeder cells (SSC vs. FSC) and expression of murine surface CD40 ligand (CD154) is shown. The histogram shaded in white represents the isotype control of the antibody.

Figure 2.

Discussion

Modifications of this protocol are possible, especially regarding the origin of CD40-stimulus and the type of mouse strain used, also yielding in efficient generation of murine CD40-activated B cells. Ahmadi et al. have shown similar results for mouse fibroblasts transfected with murine CD40-Ligand. In this context presentation of xeno-antigen can be excluded with certainty, but intensive studies regarding security and toxicity *in vivo* gave no signs for inflammation or auto-immunity reaction in this setting using human transfected HeLa cells. However, the best alternative represents a soluble murine CD40-Ligand with activating and proliferating activity at the same time, which is to our knowledge not commercially available.

This protocol also works for lymphocytes from 129S2/SvPas mice (haplotype H-2b), but has not been evaluated for further mouse strains so far. Furthermore differences in adjustment of cell density, cytokine concentration, duration of cyclosporine A treatment, cell culture media and dishes may also lead to efficient CD40-activation of murine B cells with similar proliferation rates. However, intensive work has been spent on optimizing this system maximizing activation and proliferation rates resulting in the present system.

Disclosures

The experiments were performed in accordance with the national and European guidelines for laboratory animal keeping. Referring the German animal welfare law the animals were controlled regularly by the proper authorities.

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