Cellular Immunology 256 (2009) 39–46

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

4-1BBL costimulation retrieves CD28 expression in activated T cells

Mojtaba Habib-Agahi [a,b,*], Mansooreh Jaberipour [c], Peter F. Searle [b]

[a] Immunotherapy Laboratory, Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz 71345-3119, Iran
[b] Cancer Research UK, Institute for Cancer Studies, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
[c] Shiraz Institute for Cancer Studies, Shiraz University of Medical Sciences, Shiraz, Iran

ARTICLE INFO

Article history:
Received 3 September 2008
Accepted 13 January 2009
Available online 12 February 2009

Keywords:
T cell
CD28 kinetics
CD80
CD86
CD137
4-1BBL
Costimulation

ABSTRACT

Binding of CD80/86 to CD28 is regarded as the main T cell costimulatory interaction. However, CD28 downregulates soon after T cell activation. To investigate potential cross-interaction between CD137 (4-1BB) and CD28, we stimulated T cells with anti-CD3 in the presence of A549 lung carcinoma cells expressing CD80/CD86 and 4-1BBL molecules, transduced into the cells using recombinant non-replicating adenoviruses. Following initial T cell proliferation, the proportion of $CD28^+$ cells in both $CD4^+$ and $CD8^+$ populations was rapidly reduced by CD80/86 costimulation, whereas cultures costimulated with just 4-1BBL continued to express CD28. CD28 was also downregulated in cultures costimulated with both CD80/86 and 4-1BBL. Interestingly, in cells costimulated with CD80/86 that had downregulated CD28 expression and ceased to proliferate, reactivation of proliferation by 4-1BBL costimulation also restored their CD28 expression. These findings show a positive effect of CD137 signalling on CD28 expression, similar to the effect of CD28 engagement on 4-1BB expression during the initial phases of T cell activation. Moreover, they point to the importance of signals through 4-1BB for the purposes of *ex-vivo* T cell activation and expansion.

1. Introduction

Only a few costimulatory receptors are expressed in a constitutive fashion by un-stimulated T cells. The majority of costimulatory receptors are induced only following cell activation, subsequent to signal transduction through the TCR complex. Among those receptors, there is a central role for CD28 mediated costimulation as T cell activation in the absence of CD28 engagement results in a hyporesponsive state termed anergy [1]. CD28, a homodimeric, integral membrane receptor of the B7 immunoglobulin superfamily, is known as an essential component of the immunological synapse (IS) [2–4]. The majority of human $CD4^+$ and almost half of $CD8^+$ T cells express this receptor [5]. Studies on CD28 costimulation have led to a model by which costimulators enhance TCR engagement with peptide-MHC and prolong intracellular signalling [6]. Signals mediated by CD28 potently accelerate entry into and progression through the cell cycle [7]. Triggering CD28 can initiate other signalling pathways that are distinct from the TCR but lead to the activation of common targets, such as the upregulation of anti-apoptotic members of the Bcl-2 family [8,9]. Evidence shows that reactivation of memory $CD4^+$ T cells may happen independent of the CD28 ligands, B7.1 (CD80) and B7.2 (CD86) [10]. This suggests that subsequent to initial T cell activation, a switch in the costimulatory requirement occurs. In addition, it has been shown that CD28 expression is significantly downregulated after activation in presence of CD80 costimulation [11], and occurs more rapidly in $CD8^+$ cells [12]. The $CD28^{null}$ phenotype is considered a characteristic sign of replicative senescence of human T cells during repetitive stimulation *in vitro*, as well as in chronic inflammatory and infectious disease, and in the normal course of aging [13,14]. The exact mechanism of the downregulation of CD28 in T cells is not fully understood but an inoperative transcriptional initiator (INR) at the proximal region of CD28 promoter is involved in the regulation of gene transcription [15]. Microarray analysis comparing $CD28^{null}$ and $CD28^+$ cell populations showed significantly downregulated expression of CD154 (CD40L) in $CD28^{null}$ cells. On the other hand, some other costimulatory receptors such as 4-1BB (CD137), CD244 and SLAMF7 significantly are upregulated in $CD28^{null}$ $CD8^+$ memory T cells [16]. Presence of additional costimulatory interactions could ensure the potential for continued activation of $CD28^{null}$ T cells, as these cells still are functional. Signal transduction by 4-1BB of TNFR superfamily can not only costimulate T cell activation but also have additional effects on T cell survival [17–19]. Sharing many common features with CD28, costimulation via 4-1BB has major effects on CD8 T cells and promotes TH1 differentiation/cytokine production in $CD4^+$ cells. 4-1BB is an inducible receptor and expressed on the surface of T cells after CD28 signalling and initial cell activation [20]. Therefore, we asked

* Corresponding author. Address: Immunotherapy Laboratory, Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz 71345-3119, Iran. Fax: +98 711 2351575.
E-mail addresses: agahim@sums.ac.ir (M. Habib-Agahi), habibagahim@yahoo.com (M. Habib-Agahi).

0008-8749/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2009.01.003

if 4-1BB in a similar fashion could change the pattern of CD28 expression in activated T cells. Using adenoviral vectors to express CD80, CD86 and 4-1BBL costimulatory ligands on the surface of A549 lung carcinoma cells, we showed that CD28 expression does not change during T cell activation in presence of 4-1BBL and more interestingly, CD28 that was downregulated in response to CD80/86 costimulation could be re-expressed in T cells re-activated as a result of 4-1BBL costimulation. These findings may explain possible mechanism for reactivation of $CD28^{null}$ cells and it may suggest employing other costimulatory pathways rather than CD28 for *ex-vivo* T cell proliferation.

2. Materials and methods

2.1. Expression of costimulatory ligands by adenoviral vectors

Production of E1- and E3-deleted, recombinant, non-replicative adenoviruses expressing full length human CD80, CD86, 4-1BBL and enhanced green fluorescent protein (GFP) have been reported previously [21,22]. A549 human lung carcinoma cells were infected with 300–600 virus particles/cell, 48–72 h prior to use to reach to the maximum ligand expression. Surface expression of the ligands was confirmed by flow cytometry analysis using commercial antibodies (data not shown, [22]).

2.2. Lymphocyte activation

A549 cells growing in Dulbecco's modified Eagle's medium with HEPES (DMEM-HEPES, Sigma) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 iu/ml penicillin and 100 mg/ml streptomycin, Sigma, UK) were infected with the recombinant adenoviruses to express costimulatory ligands or GFP as control, and cultured in wells of 24-well plates ($\sim$0.2–0.3 $\times$ 10^6 cells) as monolayer 48 hrs in advance. Infected cells were co-cultured with 1–1.5 $\times$ 10^6 cells/well of PBMCs prepared by Ficoll density-cushion centrifugation from blood of healthy donors (obtained with full informed consent) and depleted from plastic adherent cells. Depletion typically reduced $CD14^+$ cells from 10–15% to 3–5% of mononuclear cell preparations. Cultures were maintained in RPMI 1640 (Life Technologies, UK), supplemented with 7% FCS and 3% human (h)-AB serum (HD supplies, UK) in addition to L-glutamine and antibiotics and stimulated with 100 ng/ml soluble OKT3 anti-CD3 antibody (JANSSEN-CILAG, UK). Lymphocytes were passaged and re-stimulated approximately weekly, by transferring the adjusted number of cells to wells with fresh infected A549 cells expressing the corresponding costimulatory molecules. Lymphocyte proliferation was studied by cell surface marker staining and flow cytometry or viable cell count using hemocytometer and Trypan blue dye exclusion at the appropriate time points.

2.3. Antibody staining and flow cytomery

Fresh or cultured PBMCs were incubated with approximately 1 μg/ml of appropriate fluorochrome-conjugated or isotype matched antibodies on ice in dark for 30 min. Un-bound antibodies were removed by two washes with PBS/FCS (2%). Cells were re-suspended in 500 μl buffer for flow cytometry, and analyzed using a four colours Beckman Coulter XL flow cytometer using Coulter System II software for data acquisition and WinMDI 2.8 software for data presentation.

Labelled mouse monoclonal antibodies used in this study were sourced as follows: anti-CD4-FITC, anti-CD8-FITC, anti-CD4-PE, anti-CD8- PE, from Beckman Coulter (High Wycombe, UK), anti-CD80-FITC, anti-CD80-PE, anti-CD86-PE, anti-CD137-PE, anti-CD137L-PE, from BD, Pharmingen (Oxford, UK). Anti human CD28 antibody purchased from Sigma (UK). Appropriate isotype controls were used to indicate levels of background staining from different suppliers.

2.4. Statistical analysis

The differences between various experimental groups were analyzed by two tailed Student's *t* test, using SPSS software. Values of $p < 0.05$ were considered significant.

3. Results

3.1. CD28 expression profile in cultured lymphocytes

3.1.1. CD80/CD86 costimulation but not 4-1BBL costimulation downregulate the frequency of $CD28^+$ lymphocytes

A549 cells do not show significant expression of costimulatory ligands (0.7–1% for CD80/86 and 3–5% for 4-1BBL) however 48 h after infection with 300 p/cell of recombinant viruses, the majority

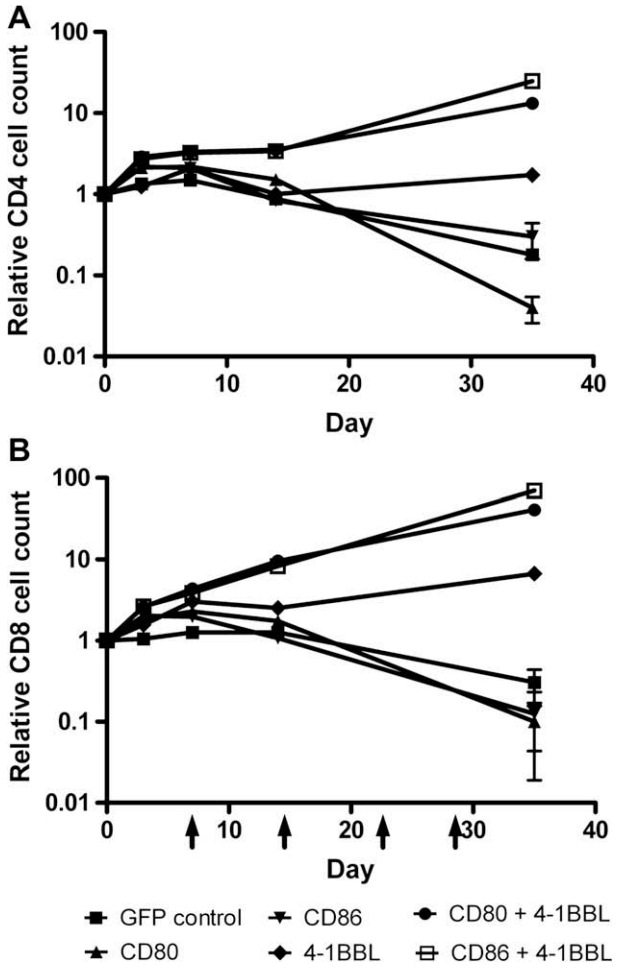

Fig. 1. Effects of different costimulation regimens on $CD4^+$ and $CD8^+$ cell numbers in long-term culture. Peripheral blood mononuclear cells were stimulated by 100 ng/ml anti-CD3 antibody whilst co-cultured with A549 lung carcinoma cells expressing one or two costimulatory transgenes (or GFP as control) as described in Section 2. Cultures were passaged, with adjustment of cell density, and re-stimulated with anti-CD3 and fresh A549 cells expressing the same co-stimulatory ligands, at weekly intervals (indicated by arrows in B). Viable cell number was determined by haemocytometer and trypan blue dye exclusion, and the proportion of $CD4^+$ (A) and $CD8^+$ cells (B) determined by flow cytometry. Cultures of lymphocytes with either CD80 or CD86 costimulation decline after 7–10 days whilst regimens including 4-1BBL allow extended lymphocyte growth. Error bars indicate standard deviation of the mean. Statistically significant differences ($p < 0.05$) determined by two-tailed *t* test.