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IFN-γ activated JAK1 shifts CD40-induced cytokine profiles in human antigen-presenting cells toward high IL-12p70 and low IL-10 production

Michael Conzelmann [a,b], Andreas H. Wagner [c], Anke Hildebrandt [c], Elena Rodionova [a,b], Michael Hess [a,b], Annika Zota [a], Thomas Giese [d], Christine S. Falk [d], Anthony D. Ho [b], Peter Dreger [b], Markus Hecker [c], Thomas Luft [a,b,*]

[a] German Cancer Research Center, Dept. of Molecular Oncology/Hematology, Heidelberg, Germany
[b] Dept. of Medicine V, University of Heidelberg, Germany
[c] Institute of Physiology and Pathophysiology, University of Heidelberg, Germany
[d] National Center for Tumor Diseases and Institute of Immunology, University of Heidelberg, Germany

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ABSTRACT

CD40Ligand (CD40L) represents a strong endogenous danger signal associated with chronic inflammatory disease. CD40L induces activation of antigen-presenting cells (APCs) such as DCs, monocytes, B-cells and endothelial cells. However, CD40 activation alone, whilst inducing IL-10 production, is insufficient to induce interleukin (IL)-12p70 release in human APCs suggesting that additional cytokine signals (e.g. GM-CSF, IL-4 or IFN-γ) are required for the induction of a pro-inflammatory cytokine profile. We demonstrate that IFN-γ-induced Janus kinase 1 (JAK1) enhances CD40-induced IL-12p70 release whilst simultaneously inhibiting IL-10 synthesis, resulting in a pro-inflammatory phenotype of CD40L-activated dendritic cells (DCs). JAK2 mediated enhancing effects on IL-12p70 but did not inhibit IL-10 release, whereas Tyk2 mediated inhibitory effects on IL-12p70 release in this system.

The mechanism by which complementary IFN-γ/JAK activities affect IL-12p70 production involves STAT1 activation and *de novo* induction of interferon-responsive factors (IRF)-1 and IRF-8. Simultaneously, JAK1 was unique in inhibiting IL-10 synthesis via STAT1 and IRF-8 with both transcription factors binding to the IL-10 promoter.

We demonstrate that CD40- and JAK/STAT/IRF-signalling pathways are strictly complementary for the induction of a pro-inflammatory cytokine profile in human APCs. This suggests that a number of CD40 effects in chronic inflammatory diseases might be weakened by targeting JAK/STAT.

1. Introduction

The CD40/CD40L system is increasingly recognized as a crucial cellular activation system of immune and non-immune cells. CD40 ligand (CD40L) induces pro-inflammatory activation of CD40-expressing antigen-presenting cells (APCs) such as dendritic cells (DCs), monocytes, B-cells and endothelial cells [1]. CD40 is a 45–50 kDa type I transmembrane protein with homology to the tumor necrosis factor (TNF) receptor [2]. In addition to its expression on the surface of immune cells such as B-cells, monocytes, macrophages and DCs, it is also found on non-immune cells such as epithelial and mesenchymal cells, haematopoietic progenitor cells, and platelets [3]. Activated endothelial cells also express CD40 together with numerous antigen-presenting features (MHC-I and -II, CD86, secretion of cytokines and chemokines), suggesting that endothelial cells are part of the antigen-presenting cell network that bridges innate and adaptive immunity [4].

CD40L is a 39 kDa type II transmembrane protein member of the TNF gene superfamily [5]. It is preferentially expressed on activated $CD4^+$ T-cells and activated platelets, however, it can also be expressed on monocytes, macrophages, DCs, natural killer cells, B-cells, $CD8^+$ T- cells, mast cells, basophils and even endothelial cells [6,7]. Following binding of CD40L, CD40 forms a trimer that triggers a signalling cascade involving binding to members of TRAFs (TNF receptor associated protein family), phosphorylation of cascades of kinases such as ERK, p38K and JNK and eventually leading to activation of transcription factors such as NF-κB, AP-1 and NFAT [8]. The best studied biological effect of CD40 signalling

* Corresponding author at: Dept. of Medicine V, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany. Tel.: +49 6221 56 8001; fax: +49 6221 56 4920.
E-mail address: thomas.luft@med.uni-heidelberg.de (T. Luft).

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is the switch in recombination and synthesis of immunoglobulins by B-cells [1,3]. The CD40 pathway also plays a significant role in a variety of immune-mediated disorders such as inflammatory bowel disease [6], systemic lupus erythematosus and rheumatoid arthritis [9,10], acute and chronic graft versus host disease (GVHD) [11], and various infectious diseases such as leprosy, leishmaniasis, and tuberculosis [12].

Cytokine receptors are multi-subunit transmembrane complexes that utilize associated tyrosine kinases of the Janus kinases (JAK) and members of the signal transducer and activator of transcription (STAT) family of transcription factors for signal transduction. Four family members have been described for the JAKs that are differentially recruited to specific cytokine receptors. For instance, the IL-4 receptor has been reported to bind JAK1, JAK2, JAK3 and Tyk2 depending on the cell type studied [13–16], whereas the GM-CSF receptor activates JAK2 and the IFN-γ receptor binds JAK1 and JAK2 [17].

We and others have reported that CD40L triggers pro-inflammatory differentiation of human monocyte-derived DCs. In particular, CD40L-induced IL-12p70 release is enhanced by cytokines such as IFN-γ, IL-4, IL-1β and IFN-α, and inhibited by PGE2/cAMP [18–24]. Strength and persistence of the CD40L activation signal also modified DC differentiation [25]. Although the interactions between CD40L and cytokines such as IFN-γ and IL-4 in the activation of human DCs have been recognized, the molecular mechanism and specifically the influence of distinct JAK family members have not yet been analysed in this context. The capacity of IL-4 to induce the release of IL-12p70 (composed of a p35 and a p40 chain coupled by two disulfide bonds [26]) is especially remarkable, as IL-12p70 is known to be one of the strongest Th1-type cytokines inducing IFN-γ as well as cytotoxic maturation of NK-cells and cytotoxic T-lymphocytes (CTLs) [26–28].

In this study we show that in a variety of human APCs, CD40L-induced IL-12p70 release strictly depends on complementary, cytokine-mediated JAK signalling. Performing siRNA knockdown studies on non-immortalized human cells, we define a so far unrecognized role for JAK1 (that was unique when compared to the other JAK family members) in the synthesis of both pro- and anti-inflammatory cytokines (IL-12p70 and IL-10).

2. Materials and methods

2.1. Media

Monocytes, DCs, peripheral mononuclear cells (PBMCs) and B-cells were cultured in complete medium: RPMI 1640 (Sigma–Aldrich, Taufkirchen, Germany) supplemented with 60 mg/l penicillin G, 12.6 mg/l streptomycin, 2 mM L-glutamine, 1% nonessential amino acids, and 10% heat-inactivated fetal calf serum (FCS; Sigma–Aldrich) in a 5% CO_2 incubator.

2.2. Monoclonal antibodies, enzyme-linked immunosorbent assays (ELISA), cytokines, chemokines and inhibitors

Flow cytometric analyses of DCs were performed using the following monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated IgG_1 isotype control; phycoerythrin (PE)-conjugated IgG_1 isotype control, PE-conjugated IgG_{2a} isotype control; anti-CD86(B70/B7-2)-PE, anti-CD83-PE, anti-CD80(B7-1)-FITC, anti-HLA-A,B,C-FITC, anti-HLA-DR-PE (BD Biosciences Pharmingen, Heidelberg, Germany). Cytokine ELISA kits (OptEIA) for IL-12p40, IL-12p70 and IL-10 were purchased from BD Pharmingen. The following cytokines were added to DC cultures: recombinant human (rh) GM-CSF (40 ng/ml, Berlex, Seattle, WA), rhIL-4 (50 U/ml; AL Immunotools, Friesoythe, Germany) and IFN-γ (1000 IU/ml; PromoCell, Heidelberg, Germany).

Cytokine secretion by stimulated MoDCs was measured by ELISA. IL-12p40, IL-12p70 and IL-10 ELISA were performed on supernatants of monocyte and MoDC cultures according to the manufacturer's instructions using Maxisorp plates (Nunc, Wiesbaden, Germany). The HRP substrate was tetramethylbenzidine (TMB) peroxidase (BD Pharmingen); the color reaction was terminated by adding 100 μl sulfuric acid (2N). Plates were read in a Sunrise microplate reader (Tecan, Salzburg, Austria).

2.3. Cell culture

PBMCs were obtained from buffy coat preparations of healthy donors from the Red Cross Blood Bank (IKTZ, Heidelberg, Germany) or from whole blood of healthy donors (after they had given their informed written consent) and used to produce MoDCs. $CD14^+$ monocytes were affinity-purified using the MACS CD14 isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) and either activated immediately or cultured at $0.5–1 \times 10^6$ cells/ml in culture medium supplemented with GM-CSF (40 ng/ml) and IL-4 (50 U/ml) in 24-well plates. By days 5–7, MoDCs represented more than 95% of cultured cells. On days 5–7, cells were washed and readjusted to $2–3 \times 10^5$ DCs per ml in cytokine-free medium. Isolation of human B-cells from whole blood of healthy donors was performed using the $CD14^-$ flow-through after $CD14^+$ affinity purification. B-cells were isolated by magnetic depletion of non-B-cells (MACS B Cell Isolation Kit II, Miltenyi Biotech).

The BHK cell line expressing CD40L was a gift of Dr. E. Leo, Dept. of Haematology/Oncology, University of Heidelberg. Expression of CD40L was confirmed by flow cytometry using an anti-CD40L mAb (BD Pharmingen), whereas the mock-transfected control BHK cell line did not express CD40L. Mock-transfected BHK cells did not induce IL-10 or IL-12p70 in human DC. The pan-JAK inhibitor I (Pyridone 6) (Calbiochem, Merck, Darmstadt, Germany) was titrated to define the maximal non-toxic concentration and added to the cell cultures 30 min prior to stimulation at a concentration of 500 nM.

2.4. Human endothelial cells

Human endothelial cells were isolated from umbilical cord veins (HUVECs) and cultured as described in detail elsewhere [29]; they were employed as a primary culture throughout. Briefly, cells were isolated from freshly collected umbilical cords by treatment with 1.6 U/ml dispase (Roche Diagnostics, Germany) for 30 min at 37 °C, and seeded into gelatin-coated 24-well plates in M199 medium containing 20% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 50 U/ml penicillin, 50 μg/ml streptomycin, 10 U/ml nystatin and endothelial cell growth supplement (Promocell, Heidelberg, Germany). They reached confluence and exhibited the typical "cobblestone" morphology after 5–6 days before commencing of the experiments. Primary HUVECs were essentially free of contaminating $CD45^+$ (<0.1%) or $CD14^+$ (<1.0%) leukocytes, as judged by FACS analysis. They were further identified by positive immunofluorescence for von Willebrand factor and negative immunofluorescence for smooth muscle α-actin.

For co-incubation experiments (24 and 48 h at 37 °C) cells were seeded into gelatin-coated 24-well plates (Greiner-Bio-One, Germany) and baby hamster kidney cells stably transfected with human CD40L (BHK-CD40L) or mock-transfected control cells were added both at 5×10^5 cells/well. As similar cytokine levels were measured in 24 and 48 h cultures, only the results of the 24-h time point are shown.

2.5. Western blot analysis

MoDCs activated for 1 h with the indicated stimuli were harvested, washed, resuspended and lysed at a density of approx.