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ERK, p38 AND NF-kappaB SIGNALING IN MONOCYTE DERIVED DENDRITIC CELLS IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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ABSTRACT

We have previously demonstrated that in patients with myelodysplastic syndrome (MDS) monocyte-derived DCs (MoDC) exhibit some phenotypic and functional abnormalities. However, the mechanisms underlying the defective response have not yet been clarified.

Aim: In the present study three different signaling pathways, ERK, p38K and NF-κB, were studied on MoDC from patients with MDS.

Materials and methods: 7 patients with MDS and 5 healthy controls were included in the study. MACS separated CD14 cells were cultured for DC generation and further stimulated with TNF-α and LPS. A migration assay and ELISA were performed for the analysis of migration and IL-12p70 secretion**.** Western blot analysis was used for the detection of the phosphorylated forms of ERK and p38K. NF-κB binding activity was evaluated by electrophoretic mobility shift assay (EMSA).

Results: In 6/7 patients the NF-κB binding activity in TNF-α and LPS stimulated MoDCs was lower compared to controls. This was accompanied by lower migratory capacity and IL-12p70 secretion. TNF-α and LPS induced phosphorylation of p38 and ERK at similar levels in control MoDC, whereas in MDS MoDC the pattern was heterogeneous with predominant activation of ERK over p38K.

Conclusion: Our results provide strong evidence that defective signaling through NF-κB and MAPK underlies the functional abnormalities of MoDC in patients with MDS.

Key words: Myelodysplastic syndromes; dendritic cells; ERK, p38, NF-κB

INTRODUCTION

Dendritic cells (DC) are sparsely distributed bone-marrow-derived cells that are continuously produced from hematopoietic stem cells. The maturation process is central to the function of the DC and enables one cell to perform different, highly specialized functions sequentially. Maturing DCs acquire the capacities to process Ag and to present it as immunogenic peptide–MHC complexes on their surface, expressing costimulatory molecules (CD54, CD56, CD80 and CD86) in parallel. Cytokine secretion is differently affected (e.g. downregulation of type I IFN and IL-10, and upregulation of IL-12) [1]. There are many stimuli that can initiate this maturation process in vitro leading to the generation of DCs with different phenotypes and stimulatory abilities, according to the intracellular pathways they activate. These include the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1b, bacterial products such as lipopolysaccharide (LPS), ligation of CD40 by CD40L and interferons [2].

Myelodysplastic syndromes (MDS) are clonal disorders of the hematopoietic stem cell characterized by ineffective hematopoiesis leading to peripheral cytopenias. Different processes are involved in its pathogenesis, such as epigenetic alterations and immunological dysfunctions. The role of DCs in the immune dysregulation in MDS has yet to be elucidated [3]. In a previous study we have demonstrated that in patients with myelodysplastic syndrome (MDS) monocyte-derived DCs (MoDC) fail to mature in the presence of TNF-α, whereas LPS activated MDS MoDC comparably to controls [4]. Different hypotheses have been proposed; however, the molecular mechanisms underlying the defective response of MDS MoDCs have not yet been clarified.

The intracellular signaling pathways implicated in maturation of MoDC are beginning to be explored. Several distinct signaling pathways such as Mitogen-activated protein kinase (MAPK) and NF-κB have been found to induce DC maturation [5]. P38 MAPK activation induced by TNF-α or LPS is necessary for expression of CD83, CD86, CD80 and HLA-DR on human monocyte-derived DC. Blocking NF-κB or p38 inhibits the maturation of DCs, indicating their pivotal role in the process [5, 6, 7]. In contrast, ERK negatively regulates the phenotypic and functional maturation of human MoDC [8].

In the present study three different signaling pathways, ERK, p38K and NF-κB, known to be implicated in MoDC maturation, were examined on TNF-α or LPS stimulated MoDC from patients with MDS and healthy controls.

MATERIALS AND METHODS

Patients

Seven patients with MDS and five healthy adults as controls were studied. Samples of heparinized blood (20ml)

were drawn at the time of diagnosis and before the administration of any treatment and from controls only once. Informed consent was obtained from both patients and normal donors. Patient data are presented in Table 1.

Table 1.

MDS case	FAB category	Sex/age (years)	Cytogenetics
1	RA	M/65	46,XY
2	RA	F/71	46,XX
3	RARS	M/79	46,XY
4	RARS	F/67	46,XX
5	RAEBI	F/75	46,XX, de5(q)
6	RAEBI	M/58	46,XY
7	CMML	M/61	46,XY

Cells and culture conditions

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation over Ficoll-Hypaque (Biochrom AG, Berlin, Germany). Peripheral blood monocytes were separated from PBMC using CD14 antibody-coated immunomagnetic beads (Miltenyi Biotec, Glodbach, Germany). CD14+ cells were cultured at a concentration of $5x10^5$ cells/ml in culture medium (CM) composed of RPMI-1640, 10% FBS, 200 mmol/l L-glutamine, 50μg/ml streptomycin, 50U/ml penicillin (all from Biochrom), 100ng/ml GM-CSF and 10ng/ml IL-4 (Biosource, Nivelles, Belgium) for 5d at 37°C in a humidified atmosphere of 5% CO_2 in 24 well tissue plate (Costar, NY, Cambridge, MA, USA). MoDCs were further cultured for 2 additional days in the presence of 10 ng/ml TNF-α (Biosource) or 0.1 μg/ml LPS (Escherichia coli, serotype 055:B6) (Sigma Chemical Co., St Louis, MO, USA).

Cytokine ELISA

IL-12p70 ELISA (Ready-SET-Go, eBioscience) was performed on supernatants from MoDCs cultures after stimulation with TNF-α or LPS. Plates were read in a Sunrise microplate reader (Tecan, Salzburg, Austria).

Migration assays

MoDCs matured with TNF-α or LPS for 48 hours were harvested from their wells, washed, and tested for migration toward CCL21 (6Ckine) chemokine using the transwell assay. Briefly, lower chambers of transwell plates (3.0 μm pore size; Costar) were filled with 500 μL RPMI/10% FBS with or without CCL21 (6Ckine; 40 ng/ml). A total of 1 x 10^4 DCs were added in 100 μL RPMI/10%FBS into the upper chamber, and cells were incubated at 37° C for 4 hours. Cells in the lower chambers were harvested, concentrated to 50 μl volumes in Eppendorf tubes, and counted with a hemocytometer. Migration for all stimulation conditions was performed in duplicate wells.

Western blot analysis

MoDCs activated for 48 hours with TNF-α or LPS were harvested, washed, resuspended at concentrations of 5 x 10^6/ml in Western sample buffer (100 mM Tris [tris(hydroxymethyl)aminomethane]–HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol), and frozen in –20° C. Prior to use, lysates were thawed, heated for 3 minutes to 96° C, and separated onto 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting. Blocking was performed in phosphate-buffered saline (PBS) plus 5% nonfat milk powder for 1 hour. Membranes were incubated with the following primary antibodies in blocking buffer plus 0.1% Tween 20 overnight at 4° C: antiphospho-ERK1/2 (Thr202/Tyr204, 1:1000; Santa Cruz Biotechnology), antiphospho-p38K (Thr180/Tyr182, 1:1000; Cell Signaling Technology, New England Biolabs, Frankfurt am Main, Germany), antitubulin (1:3000, Cell Signaling Technology). After washing, secondary antibodies were applied in blocking buffer for 1 hour at room temperature: antirabbit HRP (1:3000; Cell Signaling Technology) and antimouse HRP (1:3000; Santa Cruz Biotechnology). Membranes were washed followed by detection of immunoreactive proteins using the enhanced chemiluminescence (ECL) Western blot system (Santa Cruz Biotechnology). The exposure time was 20 minutes.

NF-κB EMSA

Electrophoretic mobility shift assay (EMSA) was performed as previously described using 10 μg cellular extract. Binding of NF-κB to 1 ng radiolabeled NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; 50 000 cpm) was induced for 20 minutes at room temperature in 10 mmol/l HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.5), 0.5 mmol/l EDTA (ethylenediaminetetraacetic acid), 100 mmol/L KCl, 2 mmol/l DTT, 2% glycerol, 4% Ficoll, 0.25% Nonidet P-40 (NP-40), 1 mg/ml bovine serum albumin (BSA), and 0.1 μg/μl poly(dIdC). Protein DNA complexes were separated from the unbound DNA probe by electrophoresis through 5% native polyacrylamide gels containing 3.25% glycerol and 0.5 x TBE (Tris-borate-EDTA). Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled NF- κB consensus oligonucleotides.

Statistical analysis

The independent data sets were compared using independent two-tailed t-tests. $P<0.05$ was considered significant.

RESULTS

Migratory capacity

MoDCs from patients with MDS showed significantly lower migratory capacity toward CCL21, compared to the controls. The mean number of migrated cells matured with TNF-α was 0.55±0.5 vs.2.4±0.7 x 10^3 and 0.73±0.6 vs. 3.75±0.6 x 10^3 after LPS stimulation (Fig.1).

Fig. 1. Migratory activity of MDS MoDCs compared to control MoDC after TNF-α and LPS stimulation (0.55±0.5 vs.2.4±0.7 x 10^3), (0.73±0.6 vs.3.75±0.6 x 10^3).

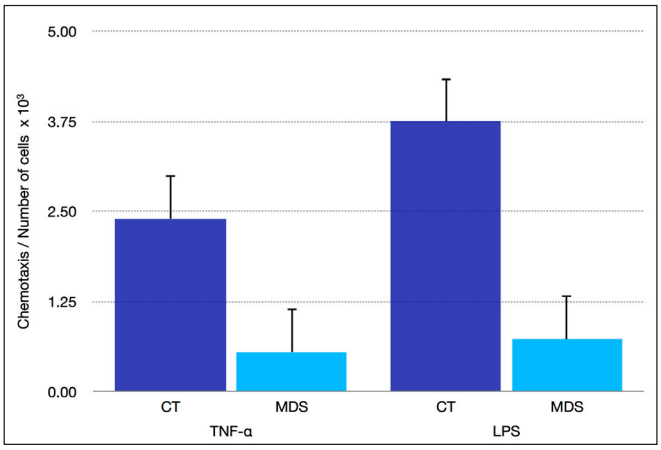
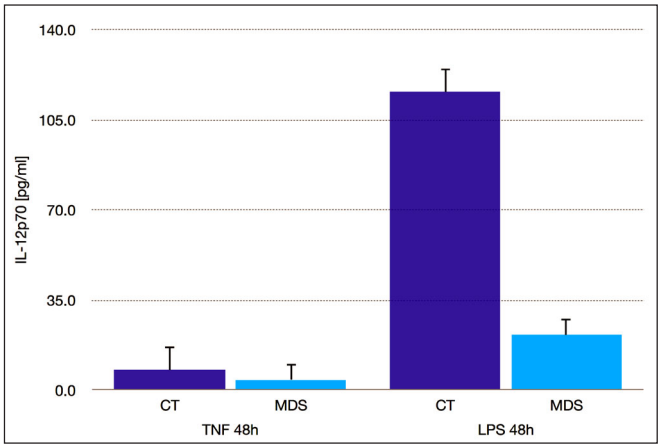


Fig. 2. Il-12p70 secretion of MDS DCs compared to controls after TNF-α and LPS stimulation.



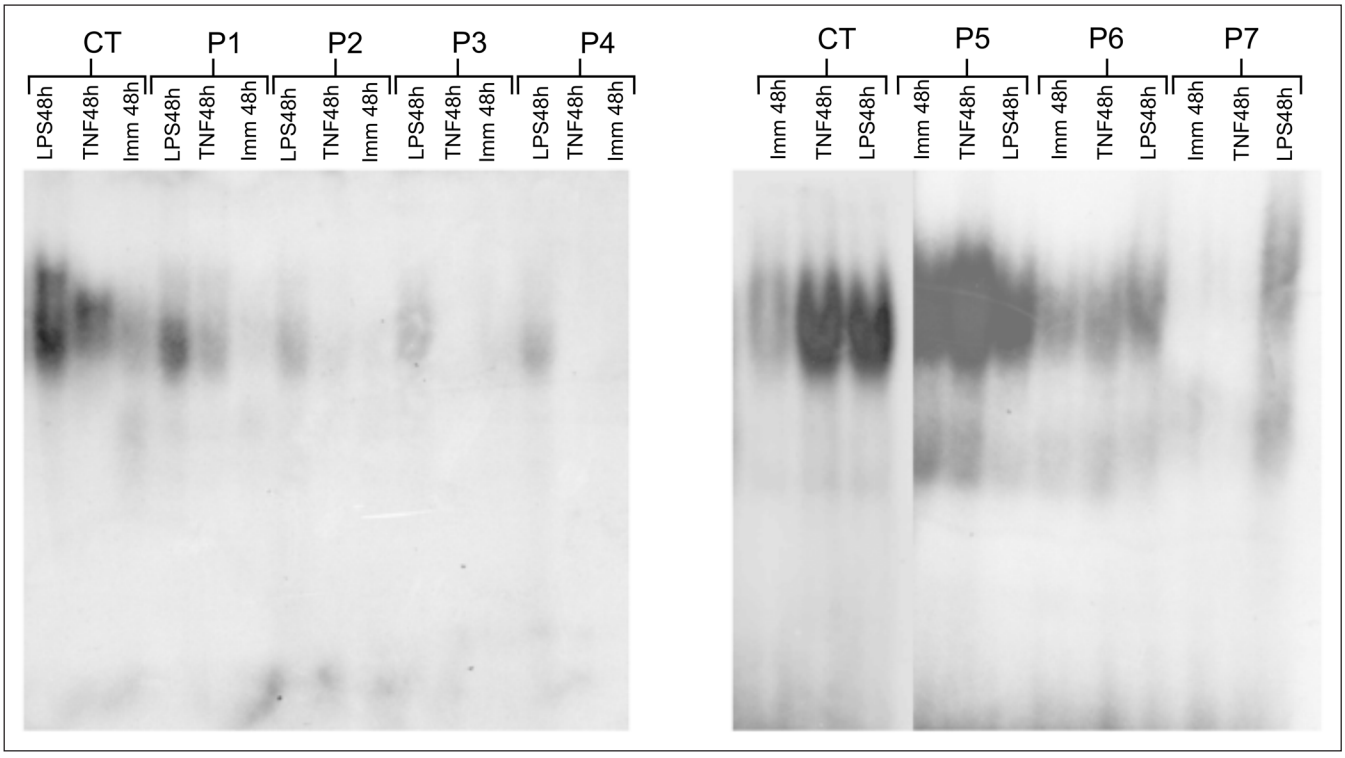
IL-12 secretion

IL-12 secretion from normal and MDS MoDCs after TNF-α stimulation was extremely low. LPS induced IL-12 secretion by both control and MDS MoDCs; however, IL-12 level in cultures of MDS MoDCs was significantly lower compared to controls (21.4±6.3pg/ml vs. 115.7±9.1pg/ml, respectively) (Fig.2).

NF-κB binding activity

NF-κB binding activity was studied on immature DCs and DCs matured with TNF-α or LPS. In 6/7 patients the NF-êB binding activity following stimulation with TNF-α for 48 hours was extremely low, whereas LPS stimulated NF-κB activity in MDS MoDC, although at lower levels compared to control MoDC (Fig.3). Interestingly, in one patient with similar to control NF-κB activity the migration and IL-12p70 production were comparable to the controls (Patient 5).

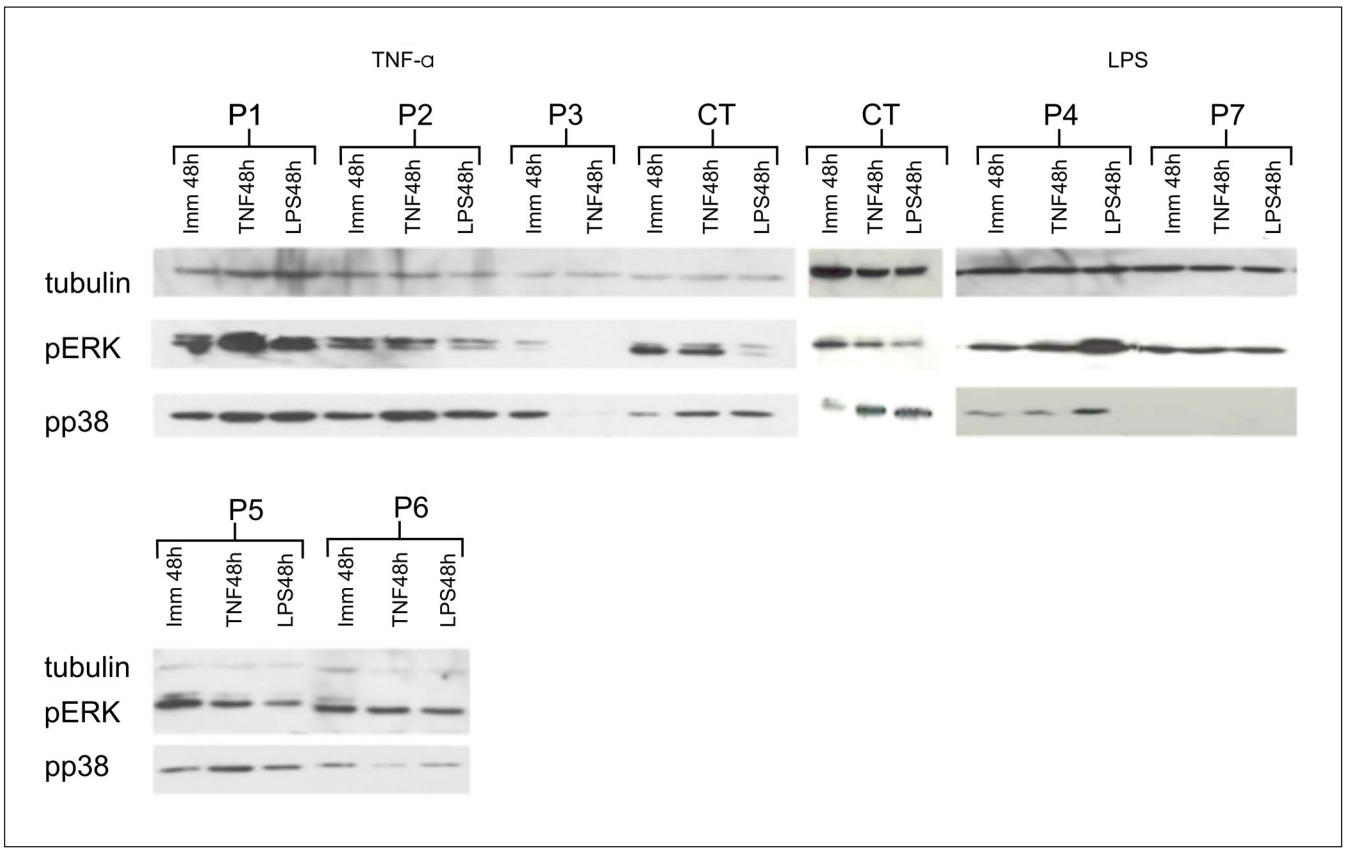
Fig. 3. NF-κB activity in MDS MoDC compared to controls after TNF-α and LPS stimulation and without stimulation (immature DCs).



Activation of p38 and ERK

Both TNF-α and LPS induced phosphorylation of p38 after 48 hours stimulation at similar levels in MoDC from controls, whereas in MDS MoDC the pattern was heterogeneous: lack of activation in Patient7 (P7), lower expression in P4, P5, P6, or normal expression in P1, P2. The phosphorylated form of ERK decreased with maturation in controls, especially after LPS stimulation. In MDS MoDCs the activation of ERK was constant and similar after TNF-α and LPS stimulation. Generally, there was predominant activation of ERK over p38K in all MDS cases (Fig.4).

Fig. 4. Phosphorylation of p38 and ERK in MDS MoDC after TNF-α and LPS stimulation and without stimulation (immature DCs), compared to controls.



DISCUSSION:

The activation of NF-κB family members is a critical control pathway for differentiation of monocytes to DCs and for maturation of DCs from antigen-processing to antigen-presenting cells. NF-κB transcription factor regulates a large number of genes involved in immune responses, such as the pro-inflammatory cytokines (IL-1, IL-6, TNF-α) and cell surface molecules, including CD80 [9].

The low NF-κB activity in MDS MoDC shows that the maturation failure of MDS MoDC, including functions such as migration and IL-12p70 secretion, is NF-κB dependent. It can be also hypothesized that the difference in MDS-MoDCs maturation by TNF-α and LPS reflects an underlying defect in the capacity of TNF-α to activate NF-κB. It has been reported that the capacity of DCs to activate T-cells following CD40L treatment was enhanced compared with TNF-α treatment, and this effect was NF-κB dependent [10].

In addition, we demonstrate a predominant activation of ERK pathway which is probably also involved in the negative regulation of MDS MoDC. ERK seems to down-regulate DC maturation, since, inhibitors of ERK enhance the phenotypical and functional maturation of MoDC and increase IL-12 production in the presence of TNF-α or LPS [8].

CONCLUSION:

Our results provide strong evidence that defective signaling through NF-κB and MAPK underlies the functional abnormalities and the differential response towards TNF-α and LPS of MoDC in patients with MDS.

REFERENCES:

1. Hart DN. Dendritic cells: Unique leukocyte populations which control the primary immune response. *Blood.* 1997 Nov 1;90(9):3245-87. [PubMed]

2. Castiello L, Sabatino M, Jin P, Clayberger C, Marincola FM, Krensky AM, et al. Monocyte-derived DC maturation strategies and related pathways: a transcriptional view. *Cancer Immunol Immunother*. 2011 Apr;60(4): 457-66. [PubMed] [CrossRef]

3. Kerkhoff N, Bontkes HJ, Westers TM, de Gruijl TD, Kordasti S, van de Loosdrec AA. Dendritic cells in myelodysplastic syndromes: from pathogenesis to immunotherapy. *Immunotherapy.* 2013 Jun;5(6):621-37. [PubMed] [CrossRef]

4. Micheva I, Thanopoulou E, Michalopoulou S, Karakantza M, Kouraklis-Symeonidis A, Mouzaki A, et al. Defective tumor necrosis factor alpha-induced maturation of monocyte-derived dendritic cells in patients with myelodysplastic syndromes. *Clin Immunology.* 2004 Dec;113(3):310-7. [PubMed] [CrossRef]

5. Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J Immunol.* 2001 Mar 15;166(6):3837-45. [PubMed] [CrossRef]

6. Ardeshna KM, Pizzey AR, Devereux S, Khwaja A. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood.* 2000 Aug 1;96(3):1039-46. [PubMed]

7. Pan K, Wang H, Liu WL, Zhang HK, Zhou J, Li JJ, et al. The pivotal role of p38 and NF-kappaB signal pathways in the maturation of human monocyte-derived dendritic cells stimulated by streptococcal agent OK-432. *Immunobiology.* 2009; 214(5): 350-8. [PubMed] [CrossRef]

8. Puig-Kröger A, Relloso M, Fernández-Capetillo O, Zubiaga A, Silva A, Bernabéu C, et al. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood.* 2001 Oct 1;98(7): 2175-82. [PubMed] [CrossRef]

9. Rescigno M, Martino M, Sutherland SL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med.* 1998 Dec 7; 188(11):2175-80. [PubMed]

10. O'Sullivan BJ, Thomas R. CD40 ligation conditions dendritic cell antigen-presenting function through sustained activation of NF-kappaB. *J Immunol.* 2002 Jun 1; 168(11):5491-8. [PubMed]

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