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CD28 ligation in the absence of TCR stimulation up-regulates IL-17A and pro-inflammatory cytokines in relapsing-remitting multiple sclerosis T lymphocytes

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ABSTRACT

CD28 is a crucial costimulatory receptor necessary full T cell activation. The role of CD28 in multiple sclerosis (MS) has been evaluated as the source of costimulatory signals integrating those delivered by TCR. However, CD28 is also able to act as a unique signaling receptor and to deliver TCR-independent autonomous signals, which regulate the expression and production of pro-inflammatory cytokines and chemokines. By comparing the cytokine/chemokine profiles of $CD4^+$ T cells from relapsing-remitting multiple sclerosis (RRMS) patients and healthy donors (HD), we found that CD28 engagement without TCR strongly up-regulates IL-8 and IL-6 expression in RRMS compared to HD. More interestingly, in RRMS but not in HD, CD28 stimulation selectively induces the expression of IL-17A by cooperating with IL-6-mediated signals. By using specific inhibitory drugs, we also identify the phosphatidylinositol 3 kinase (PI3K) as the critical regulator of CD28 proinflammatory functions in MS.

1. Introduction

The more accepted pathogenic model for multiple sclerosis (MS) is that autoreactive T cells specific for myelin-like structures entered the CNS by crossing the blood-brain barrier (BBB) [1,2], where they trigger an acute inflammatory response, thus mediating primary demyelination and axonal damage [3]. The priming and activation of autoreactive myelin-specific $CD4^+$ T cells likely occurs in peripheral lymph nodes, where the DCs may present myelin-like epitopes to näive T cells, thus inducing the activation and differentiation of autoreactive effector/memory Th1 and/or Th17 cells [4].

Two third of MS patients present the relapsing-remitting course (RRMS), which is an inflammatory phenotype of MS and is characterized by frequent relapses. These relapses are usually followed by periods of recovery or remission, but one third of patients progresses to chronic secondary progressive disease [5,6]. Pro-inflammatory cytokines and chemokines play a critical role in amplifying the inflammatory cascade by compromising the BBB, recruiting immune cells from the periphery, and activating resident microglia. Although several cell types within the CNS may contribute to the production of pro-inflammatory cytokines and chemokines, autoreactive $CD4^+$ T cells have a key role in inflammatory demyelination [3]. Thus, the identification of molecules amplifying and/or inducing the inflammatory cascade in T cells from MS patients may be pivotal for identifying new therapeutic targets.

CD28 may be considered one of the most important costimulatory receptor necessary for full T cell activation and for preventing anergy. By binding its cognate ligands B7.1/CD80 or B7.2/CD86 on the surface of professional APCs, CD28 augment TCR signaling events necessary for efficient cytokine production, cell cycle

Abbreviations: TCR, T cell receptor; APC, antigen presenting cell; MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; HD, healthy donor; PI3K, phosphatidylinositol 3 kinase; CNS, central nervous system; BBB, blood-brain barrier; DC, dendritic cells; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; NIK, NF-κB inducing kinase; IKK, IκB kinase; mTOR, mammalian target of rapamycin; CSF, cerebrospinal fluid; PIP2, phospholipids PI 3,4-biphosphate; PIP3, phospholipids PI 3,4,5-triphosphate; T_{REG}, regulatory T cells.

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progression and survival [7]. The role of CD28 in MS has been often evaluated as the source of costimulatory signals integrating those delivered by TCR [8–10]. However, CD28 is also able to act as a unique signaling receptor and to arise TCR-independent signals in primary $CD4^+$ T cells, which lead to the production of pro-inflammatory cytokine/chemokines [11,12]. No data are available on the role of CD28 as an individual signaling receptor in MS and on the molecular pathways implicated in its pro-inflammatory functions.

In the present work, we compared the cytokine and chemokine patterns of peripheral blood $CD4^+$ T cells isolated from RRMS patients and healthy donors (HD) stimulated with agonistic anti-CD28 Ab. Since RelA/NF-κB transcription factor is the unique biochemical player of CD28 individual stimulation [12], we analyzed the expression of pro-inflammatory cytokine/chemokines containing RelA/NF-κB binding sites in their promoters [13]. We found that $CD4^+$ T cells from RRMS have higher basal levels of IL-8 in comparison with HD. CD28 engagement significantly increases IL-8 production in both groups but at a higher level in RRMS. More interestingly, in RRMS but not in HD, CD28 stimulation strongly induces the expression of IL-6, IL-21 and IL-17A, all cytokines related to Th17 cells [14–18]. CD28-induced IL-17A expression requires IL-6-dependent signals. By using specific inhibitory drugs, we also demonstrate that the up-regulation of pro-inflammatory cytokines was dependent on CD28-mediated PI3K activation. Our data identify CD28 as a novel receptor molecule that may contribute to amplify the inflammatory response in RRMS by favoring pro-inflammatory cytokine production and Th17 amplification. Moreover our data suggest the PI3K signaling pathway as a novel potential therapeutic target.

2. Materials and methods

2.1. Patients and controls

30 patients with a clinically defined MS according to the McDonald criteria [19] and a clinically RRMS course [20] were enrolled from two different Centers (S. Camillo and S. Andrea Hospitals, Rome, Italy). The Ethical Committee of the Azienda Ospedaliera S. Camillo-Forlanini and the Ethical Committee of the Azienda Ospedaliera S. Andrea have approved the Study. Patients were between 27 and 59 years old (45.2 ± 8.4), had disease duration from 6 to 36 years (11.9 ± 9.1) and mild neurological disability (EDSS < 4). None of the patients had been treated with steroids or immunosuppressive agents. At follow up, all patients were in a stable phase of their disease, but four patients experienced a clinical relapse within the last year. No differences in the basal activation state or following stimulation were observed between patients not experiencing or experiencing clinical repapses within the last year. 15 age- and gender-matched healthy donor (HD) buffy coats from the blood bank of Sapienza University (Rome, Italy) with no previous history of neurological or autoimmune diseases were used as controls.

2.2. Cell isolation, Abs, and reagents

Human primary $CD4^+$ T cells were enriched from PBMCs of HD or RRMS patients by negative selection using MACS microbead sorting (Miltenyi Biotec, Milano, Italy). In some experiments $CD4^+$ T cells were isolated from PBMC by cell sorting with a MoFlo high-speed cell sorter (Beckman Coulter). No differences in T cell purity or activation state were observed by using the two methods of T cell isolation. The purity of the sorted population was 95–99%. Cells were cultured in RPMI 1640 supplemented with 5% human serum (Euroclone, UK), L-glutamine, penicillin and streptomycin.

The following antibodies were used in this study: anti-CD3 PE (clone BW264/56, Miltenyi Biotec) and anti-CD4 PC7 (Beckman Coulter, Milan, Italy) mAbs were used for T cell sorting; human anti-CD45RA-FITC (clone HI100), CD4-PerCP (clone SK3), CD27-PE (clone M-T271) and anti-CCR6 Abs (all from BD Pharmingen) were used for staining and flow cytometric analysis; human agonistic anti-CD28 (CD28.2), anti-CD3 (UCHT1), goat anti-Mouse IgG (GAM) Abs (BD Pharmingen, CA) and superagonistic CD28 ANC28.1 Ab (Ancell, MN, USA) were used for T cell stimulation; rabbit anti-PI3K pan-p85 (Upstate Biotechnology, Merck-Millipore, Italy); rabbit anti-RelA and mouse anti-PCNA Abs (Santa Cruz Biotechnology, CA, USA). Recombinant IL-6, IL-21 and neutralizing anti-IL-6R Ab (17506) were from R&D System (MN, USA). The PI3K inhibitor LY294002 (Calbiochem, Merck-Millipore), AS-605240 (Cayman Chemical, Michigan, USA), IC87114 (Symansis, New Zeland) and the mTOR inhibitor rapamycin (Calbiochem, Merck-Millipore) were used.

2.3. Cell stimulation and immunoblotting

Primary T cells were stimulated with murine L cells transfected with human B7.1 (Dap/B7) and both CD28 immunoprecipitation and nuclear extracts were prepared as previously described [11]. Proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with the indicated primary antibodies, extensively washed and after incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit or HRP-labeled goat anti-mouse (GE Healthcare, Milano, Italy), developed with the enhanced chemiluminescence's detection system (GE Healthcare).

2.4. Cell stimulation, RNA extraction and mRNA expression analysis

$CD4^+$ T cells were stimulated for the indicated times with 2 μg/ml anti-CD28.2 Ab or anti-CD3 Ab (UCHT1) crosslinked with 2 μg/ml GAM. In some experiments, exogenous recombinant IL-6 (50 ng/ml) or IL-21 (50 ng/ml) or neutralizing anti-IL-6R Ab (10 μg/ml) were added to the cultures. Total RNA was extracted using RNeasy MicroKit (Qiagen) from 5×10^5 $CD4^+$ T cells and was reverse-transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Life technologies, Milano, Italy). Real-time PCR using pre-developed TaqMan assay reagents has been used to measure the mRNA levels of the following targets: IL-8, IL-6, CXCL2, CCL2, IL-21, IL-17A, IFN-γ and the endogenous control GAPDH primers (Applied Biosystems). The relative quantification was performed using the comparative C_T method. Arbitrary units (AU) were calculated using the following formula: $2^{-\Delta\Delta CT} = [(C_{T\,\text{target gene}} - C_{T\,\text{GAPDH}}) - \Delta C_{Tcb}]$. The calibrator ΔC_{Tcb} used in all comparative analyses between HD and RRMS correspond to an unstimulated control HD value.

2.5. Cytokine production

Secretion of IL-8, IL-6 and IL-17A was measured from the supernatants of $CD4^+$ T cells cultured for 24 h (IL-8 and IL-6) or 48 h (IL-17A) in flat bottom 48 culture wells (3×10^5 cells/well) either unstimulated or stimulated with crosslinked anti-CD28.2 Ab (2 μg/ml). In the experiments with the PI3K inhibitors, cells were pretreated for 1 h with LY294002 (20 μM) or AS-605240 (10 μM) before stimulation. A cytometric bead-based immunoassay (BD CBA, BD Biosciences) was used to measure the secreted cytokines by flow cytometry (FACScalibur, BD Biosciences). Data were analyzed with the FCAP Array v3.0.1 software (Soft Flow Hungary Ltd).