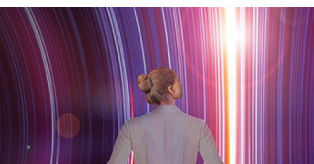


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## **IL-15 Amplifies the Pathogenic Properties of CD4<sup>+</sup>CD28<sup>-</sup> T Cells in Multiple Sclerosis**

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# IL-15 Amplifies the Pathogenic Properties of CD4<sup>+</sup>CD28<sup>−</sup> T Cells in Multiple Sclerosis

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CD4<sup>+</sup>CD28<sup>−</sup> T cells arise through repeated antigenic stimulation and are present in diseased tissues of patients with various autoimmune disorders, including multiple sclerosis (MS). These cells are believed to have cytotoxic properties that contribute to the pathogenic damaging of the target organ. Endogenous cues that are increased in the diseased tissue may amplify the activity of CD4<sup>+</sup>CD28<sup>−</sup> T cells. In this study, we focused on IL-15, a cytotoxicity-promoting cytokine that is increased in the serum and cerebrospinal fluid of MS patients. Using immunohistochemistry, we demonstrate that IL-15 is mainly produced by astrocytes and infiltrating macrophages in inflammatory lesions of MS patients. Moreover, *in vitro* transmigration studies reveal that IL-15 selectively attracts CD4<sup>+</sup>CD28<sup>−</sup> T cells of MS patients, but not of healthy individuals. IL-15 further induces the expression of chemokine receptors and adhesion molecules on CD4<sup>+</sup>CD28<sup>−</sup> T cells, as investigated using flow cytometry, resulting in enhanced migration over a monolayer of human brain endothelial cells. Finally, flow cytometric analyses revealed that IL-15 increases the proliferation and production of GM-CSF, expression of cytotoxic molecules (NKG2D, perforin, and granzyme B), and degranulation capacity of CD4<sup>+</sup>CD28<sup>−</sup> T cells. Taken together, these findings indicate that increased peripheral and local levels of IL-15 amplify the pathogenic potential of CD4<sup>+</sup>CD28<sup>−</sup> T cells, thus contributing to tissue damage in MS brain lesions. *The Journal of Immunology*, 2015, 194: 2099–2109.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS, which mainly affects young adults. MS occurs in genetically predisposed persons, carrying genetic variants that are correlated to an impaired functioning of the adaptive immune system (1, 2). In these patients, activated pathogenic Th1/Th17 cells migrate through the blood–brain barrier, contributing to the inflammatory reaction and subsequent damage to oligodendrocytes and neurons in brain lesions (3). Recently, our group identified a unique cytotoxic Th cell subset in demyelinating brain lesions of MS patients (4). These Th cells arise from repeated antigenic stimulation, after which they lose the costimulatory molecule CD28 (5, 6). CD4<sup>+</sup>CD28<sup>−</sup> T cells have cytotoxic and inflammation-seeking properties, and share many of their phenotypic features with NK cells (7). These features include expression of NK cell receptors such as NKG2D (8), intracellular stores of perforin and granzymes (9), and release of cytotoxic granules after activation (4). Expansion of CD4<sup>+</sup>CD28<sup>−</sup> T cells is associated with several autoimmune and chronic inflammatory diseases, including MS (10–12), rheumatoid arthritis (13), acute coronary syndrome (14), and myopathies (15, 16). In these dis-

eases, CD4<sup>+</sup>CD28<sup>−</sup> T cells are believed to contribute to tissue damage, but this has not been formally proved. It is worth mentioning that presently, CD4<sup>+</sup>CD28<sup>−</sup> T cells (or an equivalent subset) have not been identified in rodents.

IL-15 is a cytokine with biological functions resembling those of IL-2, although no significant homology is shared between the two (17). IL-15 was originally discovered as a T cell growth factor (17), but it additionally has important effects on NK cells (18) and monocytes (19, 20). Monocytes and dendritic cells are reported to be the main sources of IL-15 (21, 22). Both cell types transpresent IL-15, bound to the IL-15R $\alpha$ , to cells expressing the IL-15R $\beta$  and the common  $\gamma$ -chain (23). CD4<sup>+</sup> T cells express IL-15R $\beta$  (24), especially NKG2D-expressing CD4<sup>+</sup> T cells (25), which are found almost exclusively in the CD28<sup>−</sup> subset (4). Increased expression of IL-15 has been found in various inflammatory diseases, including inflammatory bowel disease (26), chronic constriction injury (27), and rheumatoid arthritis (28). In MS patients, both soluble and membrane-bound IL-15 levels are increased in the serum and cerebrospinal fluid (29–31). Recently, increased IL-15 expression was found on peripheral B cells and on astrocytes within brain lesions of MS patients, compared with control subjects (24, 32). The cytotoxicity-enhancing effect of IL-15 has already been described for NK cells and CD8<sup>+</sup> T cells (24, 32–34). Specifically, it was shown that the signaling pathways of IL-15 and the activating NK cell receptor NKG2D are intertwined (35). In this article, the effect of IL-15 on the activity of CD4<sup>+</sup>CD28<sup>−</sup> T cells of MS patients will be investigated. We hypothesize that increased peripheral and local expression of IL-15 enhances the cytotoxic properties of CD4<sup>+</sup>CD28<sup>−</sup> T cells, and thus contributes to the tissue damage in MS brain tissue.

## Materials and Methods

### Study subjects

Peripheral blood samples were collected from 12 healthy control subjects (HC) and 23 patients with clinically definite MS (14 relapsing–remitting and

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Abbreviations used in this article: CI, chemotactic index; DAB, 3,3'-diaminobenzidine; EAE, experimental autoimmune encephalomyelitis; EDSS, Kurtzke Expanded Disability Status Scale; GFAP, glial fibrillary acidic protein; HC, healthy control subject; MFI, mean fluorescence intensity; MS, multiple sclerosis.

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9 chronic progressive patients). Clinical data of patients and HCs are summarized in Table I. This study was approved by the local Medical Ethical Committee, and informed consent was obtained from all study subjects.

### Immunohistochemistry

Frozen MS brain material was obtained from the Dutch Brain Bank and subsequently cut with a Leica CM1900UV cryostat (Leica Microsystems, Wetzlar, Germany) to obtain 5- $\mu$ m sections. Slides were fixed for 10 min in acetone and dried. Slides to be stained for IL-15 using 3,3'-diaminobenzidine (DAB) were blocked in PBS containing 10% rabbit serum. Then slides were incubated overnight with goat anti-human IL-15 (diluted 1/20 in 1% rabbit serum/PBS; R&D Systems) at 4°C. After washing, slides were incubated with rabbit anti-goat HRP (diluted 1/100 in 1% rabbit serum/PBS; Dako, Glostrup, Denmark) for 1 h. Staining was performed with DAB substrate (Dako). Slides to be stained for CD3 using DAB were incubated with mouse anti-human CD3 (diluted 1/50 in 0.1% BSA/PBS; Dako) for 1 h at room temperature. After washing, slides were incubated with the Envision kit (Dako) for 30 min. Staining was performed with DAB substrate (Dako).

For immunofluorescence, slides were blocked with PBS containing 10% appropriate serum and sequentially stained for IL-15 (diluted 1/20 in 10% donkey serum/PBS; R&D Systems) and glial fibrillary acidic protein (GFAP; diluted 1/500 in 10% rabbit serum/PBS; Sigma-Aldrich, St. Louis, MO) or CD68 (diluted 1/100 in 10% rabbit serum/PBS; Dako). Secondary Abs used are, respectively, donkey anti-goat Alexa Fluor 555 (diluted 1/600 in PBS; Invitrogen) and rabbit anti-mouse biotin (diluted 1/400 in PBS; Dako) combined with streptavidin Alexa Fluor 488 (diluted 1/2000 in PBS; Invitrogen). Control staining for each Ab was performed by omitting the primary Ab. Autofluorescence was blocked by using 0.3% Sudan Black in 70% ethanol. Slides were washed thoroughly in 70% ethanol and PBS, and coverslips were applied using fluorescent mounting medium (Dako). Slides were allowed to dry before being analyzed on a Nikon eclipse 80i microscope with CFI Plan Fluor objectives (20 $\times$ : NA 0.5; 40 $\times$ : NA 0.75; 100 $\times$ : NA 1.3) and 10 $\times$  ocular lenses (all Nikon, Tokyo, Japan). Images were taken with a DS-2M-BW-C 2 megapixel camera and processed using NIS Elements BR 3.10 software (both Nikon).

### Flow cytometry

For all donors included in this study, the percentage of CD4<sup>+</sup>CD28<sup>-</sup> T cells in the peripheral blood was >2% of the CD4<sup>+</sup> T cell population to ensure that there was expansion of CD4<sup>+</sup>CD28<sup>-</sup> T cells. This was established by isolating PBMCs from whole blood by density gradient centrifugation (Histopaque; Sigma-Aldrich). Cells were double stained with anti-human CD4 FITC and CD28 PE (both BD Biosciences, Franklin Lakes, NJ) to determine the percentage of CD4<sup>+</sup>CD28<sup>-</sup> T cells. In all flow cytometric experiments, lymphocytes were gated using forward and side scatter parameters, after which CD4 and CD28 expression were monitored within the lymphocyte gate.

For phenotypical characterization experiments, PBMCs of HCs (cytotoxic markers:  $n = 9$ ; adhesion/migration markers:  $n = 4$ ) and MS patients (cytotoxic markers:  $n = 12$ , 10 relapsing-remitting and 2 chronic-progressive patients with Kurtzke Expanded Disability Status Scale [EDSS] scores between 2 and 6.5; adhesion/migration markers:  $n = 6$ , 5 relapsing-remitting and 1 chronic-progressive patient with EDSS score between 2 and 6.5) were analyzed directly *ex vivo*, and subsequently seeded in 96-well round-bottom plates (Nunc, Roskilde, Denmark) at  $2 \times 10^5$  cells/well in culture medium, consisting of RPMI 1640 medium (Lonza, Basel, Switzerland), supplemented with 10% heat-inactivated FBS (Life Technologies, Carlsbad, CA), 1% sodium pyruvate, 1% nonessential amino acids, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin (all Invitrogen, Carlsbad, CA). Cells were stimulated with 10 ng/ml recombinant human IL-15 (R&D Systems, Minneapolis, MN) and cultured at 37°C/5% CO<sub>2</sub>. As a control condition, culture medium without addition of IL-15 was used. *Ex vivo*, and after 72 h of culture, cells were stained with anti-human CD4 PerCP or CD4 PE-CF594, CD28 FITC, CD28 allophycocyanin, or CD28 PE (all BD Biosciences) in combination with the following Abs: NKG2D PE, perforin PE, granzyme B FITC, CXCR5 AF488, CD54 PE (all BD Biosciences), CCR1 PE (R&D Systems), CCR7 PE (eBioscience, San Diego, CA), CX3CR1 PE (MBL International, Woburn, MA), CD11a FITC (Biolegend, San Diego, CA), CD49d FITC, IFN- $\gamma$  PE (both Immunotools, Friesoythe, Germany). For intracellular molecules, cells were first fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Samples were analyzed by flow cytometry using a FACSCalibur and CellQuest software or FACSARIA II and Diva software (all BD Biosciences).

For analysis of cytokine production, PBMCs of 3 HCs and 4 MS patients (4 relapsing-remitting patients with EDSS scores between 2.5 and 4) were stimulated with 10 ng/ml recombinant human IL-15 (R&D Systems), 2  $\mu$ g/ml anti-CD3 (clone 2G3; BIOMED, Diepenbeek, Belgium), or the combination

of both, and cultured at 37°C/5% CO<sub>2</sub> for 6 d. During the last 5 h, cells were activated with 20 ng/ml PMA and 0.5  $\mu$ g/ml calcium ionomycin (both Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). After activation, cells were surface stained with anti-human CD4 PE-CF594, CD28 PE (both BD Biosciences), and stained intracellularly with the Abs GM-CSF AF647 and IL-17A FITC (both eBioscience). Cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and analyzed by flow cytometry using FACSARIA II and Diva software (both BD Biosciences).

ICAM and VCAM expression were determined on the human brain endothelial cell line hCMEC/D3 (36) after stimulation with 5 ng/ml TNF- $\alpha$  (24 h; Peprotech), 10 ng/ml IL-15, or 50 ng/ml IL-15 (both 72 h; R&D Systems). Cells were surface stained for ICAM (Rek-1; 5  $\mu$ g/ml, a kind gift from the Department of Tumor Immunology, University Medical Center St. Radboud, Nijmegen, The Netherlands) and VCAM (AbD Serotec, Oxford, U.K.). Binding was detected using goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR). Samples were analyzed on a FACSCalibur using CellQuest Pro software (both BD Biosciences).

### Quantitative PCR

For analysis of IL-15 expression on astrocytes, primary astrocytes from MS lesions were isolated and cultured as described previously (37, 38). To mimic inflammation in the brain of MS patients, we stimulated astrocytes with TNF- $\alpha$  (5 ng/ml; Peprotech, Rocky Hill, NJ) during 24 h. mRNA was isolated using an mRNA capture kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized with the Reverse Transcription System kit (Promega, Fitchburg, WI) following the manufacturer's guidelines, and real-time quantitative PCR was performed as described previously (39). IL-15 primer sequences are 5'-CCAACCTGGGTGAATGTAATAAGTGA-3' (forward) and 5'-TGCAACTGGGGTGAACATCA-3' (reverse). Expression levels of transcripts were normalized to GAPDH expression levels.

### Chemotaxis assay

PBMCs of four HCs and five MS patients (three relapsing-remitting and two chronic-progressive patients with EDSS scores between 2 and 6.5) were stained with anti-human CD4 FITC and CD28 PE Abs (both BD Biosciences), after which CD4<sup>+</sup> T cells were sorted using a FACSARIA II (BD Biosciences). The purity of the sorted cell population was routinely >95%. A transwell system with a pore size of 5  $\mu$ m (Corning, Lowell, MA) was used for the migration assay. In the bottom compartment, IL-15 (R&D Systems) was added to chemotaxis buffer (0.5% BSA; US Biological, Swampscott, MA)/RPMI 1640 (Lonza) in increasing concentrations (0, 0.1, 1, 10 ng/ml). In each insert,  $5 \times 10^5$  CD4<sup>+</sup> T cells were seeded. After 4 h of incubation at 37°C/5% CO<sub>2</sub>, the total number of cells that migrated to the bottom compartment was counted. Using flow cytometry on a FACSARIA II (BD Biosciences), the percentages of both CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>-</sup> T cells in the migrated fraction were determined. The chemotactic index (CI) of both cell populations and for each IL-15 concentration was calculated as follows: (number of migrated cells with IL-15)/(number of migrated cells without IL-15).

### Time-lapse migration assay

PBMCs of two HCs were seeded in 100-mm diameter petri dishes (Nunc) at a concentration of  $2 \times 10^6$ /ml and stimulated with 10 ng/ml IL-15 (R&D Systems) or left untreated. After 72 h, cells were harvested and surface stained with CD4 FITC and CD28 PE (both BD Biosciences), after which CD4<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>+</sup> T cells were sorted using a FACSARIA II (BD Biosciences). After sorting, cells were left to rest overnight in culture medium with or without 10 ng/ml IL-15. The next day,  $6 \times 10^4$  cells were added to a brain endothelial cell line monolayer (hCMEC/D3 cells) (36) cultured in the presence of 5 ng/ml TNF- $\alpha$  for 24 h in 96-well, flat-bottom plates (Greiner Bio-One, Wemmel, Belgium), after washing of both cell types. For each condition, a minimum of three wells was used. T cells were then allowed to migrate during 4 h, after which the cocultures were imaged using time-lapse microscopy, as described by Reijerkerk et al. (40). In brief, cocultures were placed in an inverted phase-contrast microscope (Nikon Eclipse TE300; Nikon, Leiden, The Netherlands) housed in a temperature-controlled (37°C), 5% CO<sub>2</sub> gassed chamber. A field (220  $\times$  220  $\mu$ m) was randomly selected and recorded for 10 min (55 single pictures) using a digital video camera and Cell F imaging software (Olympus, Heidelberg, Germany). T cell transmigration was assessed by enumerating the number of T cells within the field that had either adhered to or migrated through the monolayer. Transmigrated T cells (phase-dark) could be readily distinguished from those remaining on the surface by their highly refractive (phase-bright) morphology.



### Resistance measurement (ECIS)

ECIS Model 1600R (Applied BioPhysics) was used to measure the transendothelial electric resistance of hCMEC/D3 cell monolayers in real time as described previously (41). A total of  $1 \times 10^5$  endothelial cells/well was seeded into an 8W10+ ECIS array coated with collagen. Impedance was measured at 4000 Hz in real time. Cells were treated with 5 ng/ml TNF- $\alpha$  (after formation of barrier; Peprotech), 10 ng/ml IL-15, or 50 ng/ml IL-15 (both 24 h after seeding; R&D Systems).

### Proliferation assay

CFSE-labeled (2  $\mu$ M; Molecular Probes/Invitrogen) PBMCs of 10 HCs and 10 MS patients (8 relapsing-remitting and 2 chronic-progressive patients with EDSS scores between 2 and 6.5) were seeded in 96-well, round-bottom plates (Nunc) at  $2 \times 10^5$  cells/well in culture medium. Cells were stimulated with anti-CD3 (2  $\mu$ g/ml; clone 2G3; BIOMED), IL-2 (5 U/ml; Roche Diagnostics), IL-15 (10 ng/ml; R&D Systems), anti-CD3+IL-2, or anti-CD3+IL-15 and cultured at 37°C/5% CO<sub>2</sub>. After 5 d, cells were labeled with anti-human CD4 PerCP and CD28 PE (both BD Biosciences), and analyzed by flow cytometry using FACSCalibur and CellQuest software (both BD Biosciences) to determine the percentage of proliferating cells (CFSE<sup>low</sup> cells within CD4<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>+</sup> gates).

### Degranulation assay

PBMCs of eight HCs and seven MS patients (six relapsing-remitting and one chronic-progressive patient with EDSS scores between 2.5 and 6.5) were seeded in 96-well, round-bottom plates (Nunc) at  $2 \times 10^5$  cells/well in culture medium. Cells were either stimulated with 10 ng/ml IL-15 (R&D Systems) or left unstimulated. Percentage degranulation was measured directly ex vivo and daily for 3 d. Four hours before the analysis, the following substances were added to the culture to provoke degranulation: anti-human CD107a PE, GolgiPlug (both BD Biosciences), and anti-CD3 (BIOMED). Flow cytometry was performed by labeling cells with anti-human CD4 PerCP and CD28 PE (both BD Biosciences). Samples were analyzed using FACSCalibur and CellQuest software (both BD Biosciences).

### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4.03. Two-tailed Student *t* test (with Welch's correction if necessary) was performed to compare two groups. A *p* value <0.05 was considered significant. Results are expressed as mean value  $\pm$  SEM.

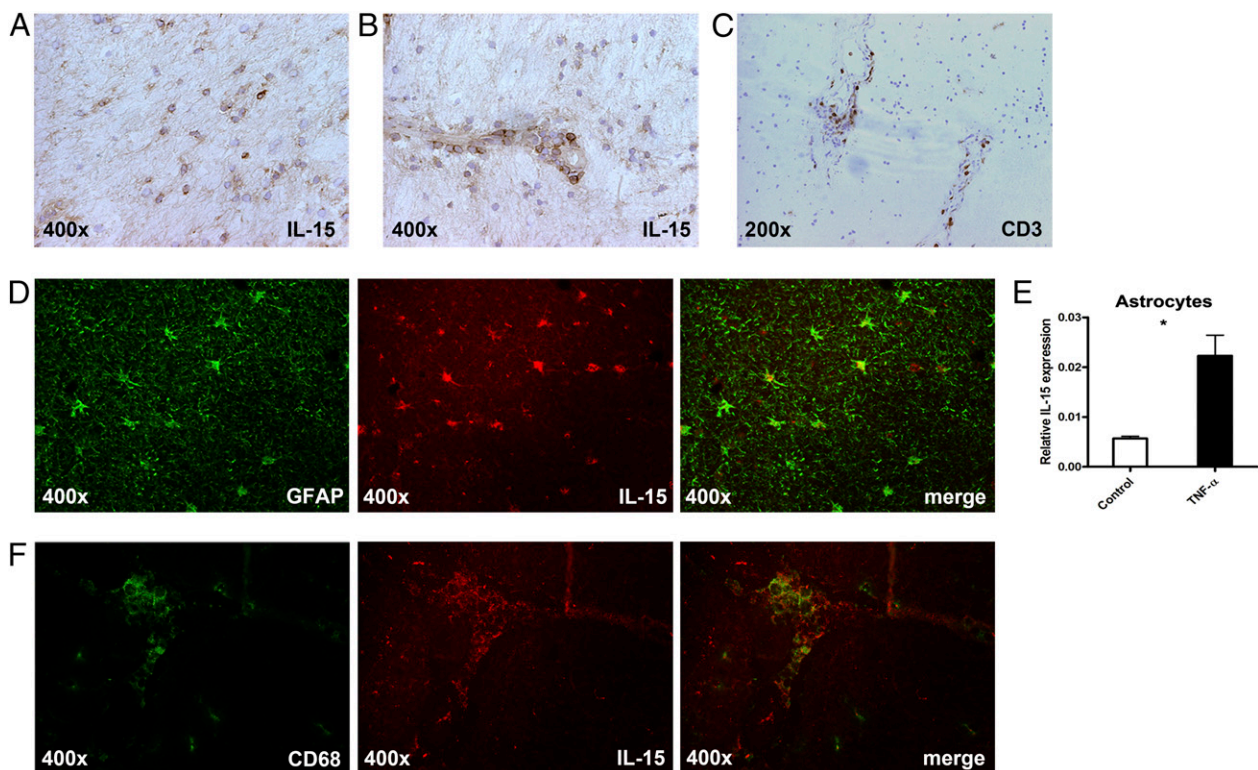
## Results

### IL-15 is produced by reactive astrocytes and infiltrating macrophages in MS brain lesions

In the CNS of MS patients, astrocytes were shown to be an important source of IL-15 (32). In this study, we confirm that astrocytes express IL-15 inside brain lesions (Fig. 1A, 1D) and in vitro after TNF- $\alpha$  stimulation (Fig. 1E). In addition, within perivascular cuffs, IL-15<sup>+</sup> infiltrating immune cells were found (Fig. 1B). The staining pattern revealed positivity on the membranes of the cells, indicating that the membrane-bound form of IL-15 is expressed, suggesting transpresentation of the cytokine. Although the perivascular infiltrates partly consist of CD3<sup>+</sup> T cells (Fig. 1C), flow cytometry revealed that PMA-stimulated human T cells do not express IL-15. We found that CD68<sup>+</sup> infiltrating immune cells express IL-15 (Fig. 1F). Because we found these cells within the perivascular region, we conclude that these cells are macrophages, rather than microglia. In summary, these findings suggest that astrocytes and infiltrating macrophages present IL-15 to T cells inside the CNS of MS patients.

### IL-15 selectively attracts CD4<sup>+</sup>CD28<sup>-</sup> T cells of MS patients and enhances their migratory capacity

Previously, we reported that CD4<sup>+</sup>CD28<sup>-</sup> T cells are potentially pathogenic in MS patients (4). In these patients, IL-15 levels are

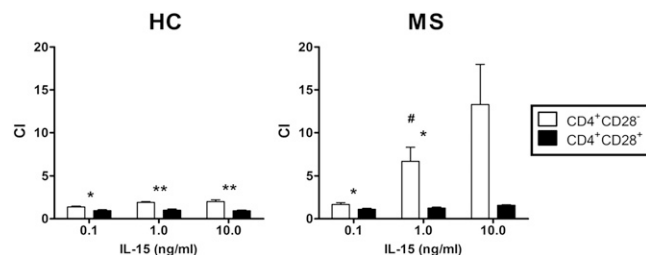


**FIGURE 1.** Astrocytes and infiltrating immune cells express IL-15 in brain lesions. MS brain tissue was stained for IL-15 (A, B, D, and F), GFAP (D), CD3 (C), and CD68 (F). (A) In the brain parenchyma, cells with astrocyte morphology stain positive for IL-15 (brown). (B) A perivascular cuff shows IL-15<sup>+</sup> infiltrating immune cells (brown). (C) The perivascular cuffs contain CD3<sup>+</sup> T cells (brown). (D) Immunofluorescence reveals that GFAP<sup>+</sup> astrocytes (green) express IL-15 (red). (E) Quantitative PCR was performed on in vitro TNF- $\alpha$ -stimulated primary human astrocytes to determine expression of IL-15. (F) Immunofluorescence reveals that CD68<sup>+</sup> infiltrating cells (green) express IL-15 (red). Original magnification  $\times 400$  (A, B, D, and F),  $\times 200$  (C). \**p* < 0.05.

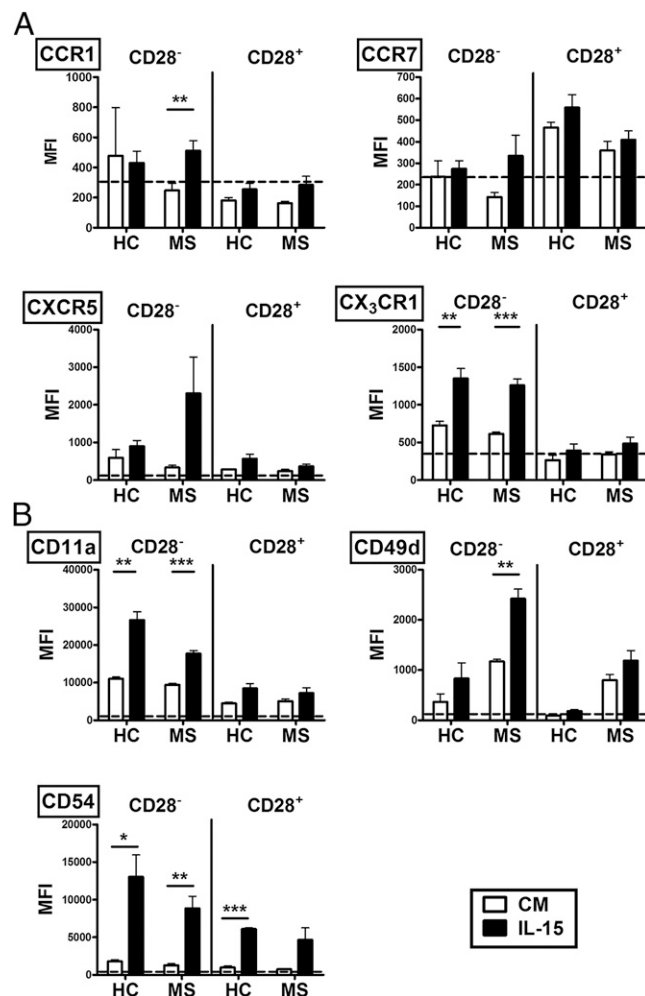
increased in serum and cerebrospinal fluid (29–31). In line with previous reports (24, 25), we observed that IL-15R $\beta$  is expressed on a subset of CD4<sup>+</sup> T cells and that IL-15R $\beta$  mRNA is increased in CD4<sup>+</sup>CD28<sup>-</sup> T cells compared with CD4<sup>+</sup>CD28<sup>+</sup> T cells without differences between MS patients and HCs (data not shown). Next, we performed a transwell migration assay using increasing concentrations of IL-15 to determine the migration of CD4<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>+</sup> T cells toward the cytokine. We found that IL-15 has a low but significant migration-inducing effect on CD4<sup>+</sup>CD28<sup>-</sup> T cells of HCs at a concentration of  $\geq 0.1$  ng/ml ( $1 < CI < 2$ ), whereas no effect on CD4<sup>+</sup>CD28<sup>+</sup> T cells was observed (Fig. 2). When cells of patients (Table I) were used, we found a remarkable enhancement in migration of CD4<sup>+</sup>CD28<sup>-</sup> T cells toward IL-15 ( $3 < CI < 30$ ), whereas again CD4<sup>+</sup>CD28<sup>+</sup> T cells of MS patients did not display specific migration to IL-15 (Fig. 2). The difference between migration of control- and patient-derived CD4<sup>+</sup>CD28<sup>-</sup> T cells reached significance at a concentration of 1 ng/ml. This was not the result of differences in ex vivo frequency of CD4<sup>+</sup>CD28<sup>-</sup> T cells (HC:  $5.3 \pm 2.2\%$  versus MS:  $5.7 \pm 0.7\%$ ;  $p = 0.85$ ). Furthermore, the migration potential of CD4<sup>+</sup>CD28<sup>-</sup> T cells was not affected by either disease type or treatment of MS patients. In fact, we did not observe any effect of clinical MS parameters on any phenotypical or functional characteristic of CD4<sup>+</sup>CD28<sup>-</sup> T cells in this study and in our previous study (4).

To explain the altered migratory behavior, we next determined the effect of IL-15 on expression of chemokine receptors and adhesion molecules. We found that CCR1 and CX<sub>3</sub>CR1 were significantly induced on CD4<sup>+</sup>CD28<sup>-</sup> T cells after 72 h of IL-15 treatment (Fig. 3A), whereas expression of CXCR5 and CCR7 was not significantly induced. The increase in CCR1 was specific for cells of MS patients, whereas CX<sub>3</sub>CR1 expression was increased on cells of both HCs and patients. In contrast, these chemokine receptors were not changed on CD4<sup>+</sup>CD28<sup>+</sup> T cells in response to IL-15. CX<sub>3</sub>CR1 was previously shown to be expressed on CD4<sup>+</sup>CD28<sup>-</sup> T cells, whereas being almost absent on CD4<sup>+</sup>CD28<sup>+</sup> T cells. Our group recently found that CD4<sup>+</sup>CD28<sup>-</sup> T cells use this receptor to migrate via a CX<sub>3</sub>CL1 gradient to the CNS of MS patients (4). We now show that the expression of CX<sub>3</sub>CR1 can be increased selectively on CD4<sup>+</sup>CD28<sup>-</sup> by IL-15 (Fig. 3A).

In addition to chemokine receptors, we also describe that IL-15 induces the expression of the integrins CD11a (part of LFA-1), CD49d ( $\alpha 4$  integrin), and the adhesion molecule CD54 (ICAM-1) on CD4<sup>+</sup>CD28<sup>-</sup> T cells (Fig. 3B). For CD49d, this increase on CD4<sup>+</sup>CD28<sup>-</sup> T cells was only evident in MS patients. The expression of CD49d was significantly higher in patients compared



**FIGURE 2.** IL-15 specifically chemoattracts CD4<sup>+</sup>CD28<sup>-</sup> T cells of MS patients. Sorted CD4<sup>+</sup> T cells of four HCs and five MS patients were allowed to migrate in a transwell system toward different concentrations of IL-15 (0–10 ng/ml). Cells were analyzed by flow cytometry to determine the number of migrated CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>-</sup> T cells. The CI was calculated as follows: (number of migrated cells with IL-15)/(number of migrated cells without IL-15). CI of CD4<sup>+</sup>CD28<sup>-</sup> T cells was compared with CI of CD4<sup>+</sup>CD28<sup>+</sup> T cells. Graphs represent mean values of all donors. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.05$  between HCs and patients with MS.

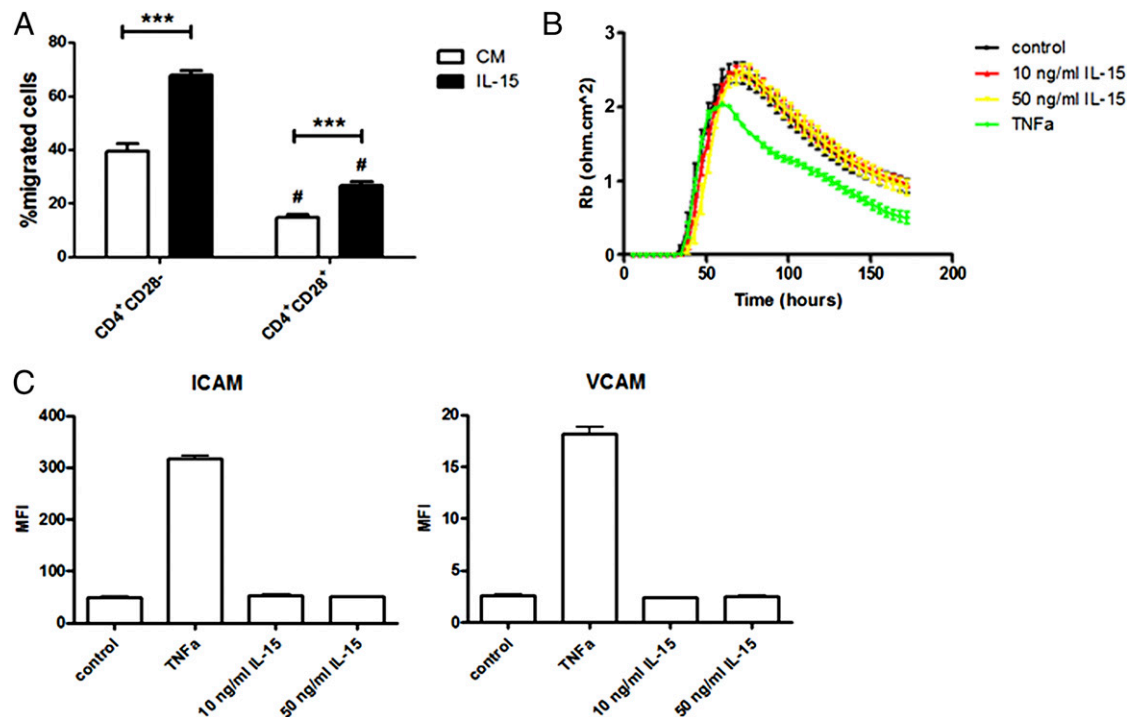


**FIGURE 3.** IL-15 increases the expression of chemokine receptors and adhesion molecules on CD4<sup>+</sup>CD28<sup>-</sup> T cells. PBMCs of four HCs and six MS patients were stimulated with IL-15 (10 ng/ml) or left untreated for 72 h. Flow cytometry was performed to determine the expression of CCR1, CCR7, CXCR5, and CX<sub>3</sub>CR1 (A) and CD11a, CD49d, and CD54 (B). The MFI of each marker within CD4<sup>+</sup>CD28<sup>-</sup> or CD4<sup>+</sup>CD28<sup>+</sup> cells is shown. Dotted line is the MFI of the isotype controls. Graphs represent mean values of all donors. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  between CD4<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>+</sup> T cells (both conditions). CM, culture medium.

with HCs in all conditions ( $p < 0.01$ ). In MS patients, CD49d expression was highest on CD4<sup>+</sup>CD28<sup>-</sup> T cells ( $p < 0.05$ ). The expression of CD11a was at a lower level on CD4<sup>+</sup>CD28<sup>-</sup> T cells of patients compared with HCs, whereas CD4<sup>+</sup>CD28<sup>-</sup> T cells were still the highest expressing cells.

For these experiments, the total PBMC fraction was treated with IL-15, because transpresentation of IL-15 by monocytes might be a possible activation route. However, experiments on sorted CD4<sup>+</sup>CD28<sup>-</sup> T cells show that IL-15 also directly affects these cells without the need of bystander cells (data not shown). Furthermore, we found that IL-15 does not induce loss of CD28 in our cultures, thus indicating that the expression of molecules in CD4<sup>+</sup>CD28<sup>-</sup> T cells are not contaminated by in vitro-induced CD28<sup>-</sup> T cells (data not shown).

Next, we investigated whether IL-15 treatment could enhance migration across a TNF- $\alpha$ -activated human brain endothelial monolayer, mimicking the inflamed blood–brain barrier as seen in MS patients. CD4<sup>+</sup>CD28<sup>-</sup> T cells ( $39.5 \pm 2.7\%$ ) readily migrated across the brain endothelial layer, which was  $>2$ -fold higher than the percentage of migrated CD4<sup>+</sup>CD28<sup>+</sup> T cells ( $14.8 \pm 1.2\%$ ;  $p < 0.001$ ). Treatment of both T cell subsets with IL-15 before the



**FIGURE 4.** IL-15 increases migration of CD4<sup>+</sup>CD28<sup>-</sup> T cells across a human brain endothelial monolayer. **(A)** Migration of unstimulated and IL-15-stimulated sorted CD4<sup>+</sup>CD28<sup>-</sup> (left) and CD4<sup>+</sup>CD28<sup>+</sup> (right) T cells of two HCs across a monolayer of human brain endothelial cells was measured using a time-lapse migration experiment. The percentage of migrated cells was calculated as follows: (number of cells below monolayer surface)/(number of cells on monolayer surface + number of cells below monolayer surface). Graphs represent mean values of all donors. **(B)** Resistance measurements on a monolayer of hCMEC/D3 cells treated with TNF-α or IL-15 show that only TNF-α treatment increases permeability. **(C)** Flow cytometry of TNF-α- or IL-15-treated hCMEC/D3 cells reveals that only TNF-α induces upregulation of VCAM and ICAM. \*\*\**p* < 0.001, #*p* < 0.001 between CD4<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>+</sup> T cells (both conditions). CM, culture medium.

start of the experiment significantly enhanced their migratory potential by almost 2-fold: CD4<sup>+</sup>CD28<sup>-</sup> T cells to 67.9 ± 1.5% and CD4<sup>+</sup>CD28<sup>+</sup> T cells to 26.7 ± 1.3% (*p* < 0.001; Fig. 4A).

To rule out that IL-15 directly affected the endothelial monolayer, we monitored the expression of ICAM and VCAM after treating the endothelial cells with IL-15. In contrast with TNF-α, IL-15 did not induce expression of both adhesion markers (Fig. 4C). Furthermore, resistance measurements revealed that only TNF-α and not IL-15 reduced endothelial monolayer integrity (Fig. 4B).

Taken together, these data suggest that IL-15 itself is strongly chemotactic to CD4<sup>+</sup>CD28<sup>-</sup> T cells of MS patients. Furthermore, it has a striking effect on the expression of chemokine receptors and adhesion molecules on these cells. IL-15 therefore enhances the migratory potential of CD4<sup>+</sup>CD28<sup>-</sup> T cells across an activated human brain endothelial monolayer.

*IL-15 induces proliferation of CD4<sup>+</sup>CD28<sup>-</sup> T cells*

IL-15 was originally identified as a T cell growth factor (17). In this study, we analyzed the effect of IL-15 on the proliferation of CD4<sup>+</sup>CD28<sup>-</sup> T cells from HC and MS patients with a CFSE-based proliferation assay. We found that, for each condition tested, CD4<sup>+</sup>CD28<sup>-</sup> T cells displayed a higher proliferative capacity compared with their CD28<sup>+</sup> counterparts (Fig. 5A). Within the CD4<sup>+</sup>CD28<sup>-</sup> T cell population, IL-15 treatment alone induced proliferation of these cells, whereas IL-2 did not (Fig. 5A, 5B). Most proliferation was seen when cells were stimulated with anti-CD3 in combination with IL-15 (HC: 85.5 ± 3.3%; MS: 84.8 ± 4.4%; Fig. 5A, 5B). Interestingly, this combination induced preferential proliferation of CD4<sup>+</sup>CD28<sup>-</sup> T cells, because these were all found in the CFSE<sup>low</sup> population. This was in contrast with the CD28<sup>+</sup> subset, which only partially proliferated in re-

Table I. Study subjects used for analysis of CD4<sup>+</sup>CD28<sup>-</sup> T cells in the peripheral blood

|                             | Total MS Group (n = 23) | RR (n = 14)  | CP (n = 9)   | HCs (n = 12) |
|-----------------------------|-------------------------|--------------|--------------|--------------|
| Age (range), y              | 45.5 (29–71)            | 43.5 (29–58) | 48.7 (39–71) | 38.6 (25–58) |
| Female/Male (ratio), n      | 16/7 (2.3)              | 10/4 (2.5)   | 6/3 (2)      | 7/5 (1.4)    |
| Disease duration (range), y | 11.7 (1–34)             | 7.5 (1–27)   | 18.4 (2–34)  | —            |
| EDSS score (range)          | 3.7 (0–7)               | 2.2 (0–5)    | 5.2 (3–7)    | —            |
| Treated, <sup>a</sup> n     |                         |              |              |              |
| None                        | 7                       | 3            | 4            | —            |
| IFN-β                       | 3                       | 2            | 1            | —            |
| Natalizumab                 | 2                       | 2            | 0            | —            |
| Cyclophosphamide            | 1                       | 0            | 1            | —            |
| GA                          | 5                       | 3            | 2            | —            |
| MTX                         | 1                       | 0            | 1            | —            |
| Fingolimod                  | 4                       | 4            | 0            | —            |

<sup>a</sup>Within 3 mo before blood sampling.  
—, not applicable; CP, chronic-progressive; GA, glatiramer acetate; MTX, mitoxantrone; RR, relapsing-remitting.



sponse to anti-CD3 stimulation combined with IL-15 (CFSE<sup>low</sup> CD28<sup>+</sup> cells in HCs: 33.3 ± 6.0%; MS patients: 58.4 ± 3.7%). Combined, these results demonstrate that IL-15 (in combination with anti-CD3 stimulation) preferentially induces proliferation of CD4<sup>+</sup>CD28<sup>-</sup> T cells.

#### IL-15-stimulated CD4<sup>+</sup>CD28<sup>-</sup> T cells produce GM-CSF

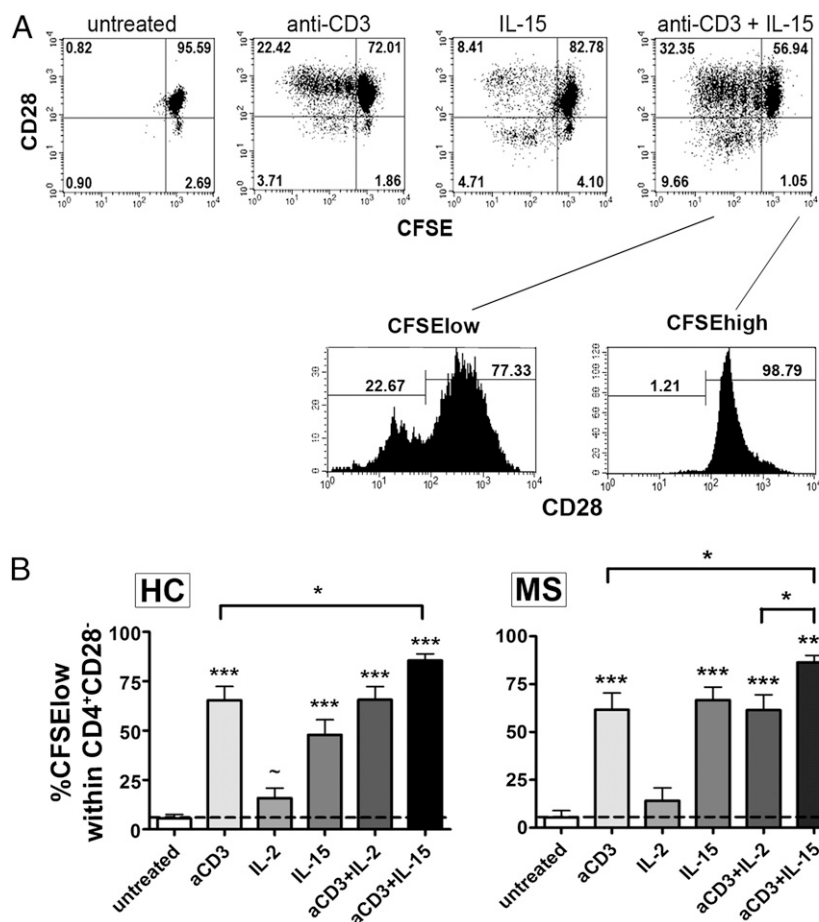
To identify whether IL-15 can induce or upregulate production of disease-relevant proinflammatory cytokines by CD4<sup>+</sup>CD28<sup>-</sup> T cells, we performed intracellular flow cytometry. We chose to investigate two cytokines relevant for MS, namely, GM-CSF and IL-17A. GM-CSF has been shown to be crucial for the induction of experimental autoimmune encephalomyelitis (EAE) by Th cells (even when lacking expression of IFN-γ and IL-17A) (42). We found that CD4<sup>+</sup>CD28<sup>-</sup> T cells produce GM-CSF, but not IL-17A, after stimulation with IL-15, either alone or in combination with anti-CD3 (Fig. 6A). Anti-CD3 stimulation alone did not result in significant GM-CSF production by CD4<sup>+</sup>CD28<sup>-</sup> T cells, in contrast with CD4<sup>+</sup>CD28<sup>+</sup> T cells (Fig. 6B). Also, IL-17A was not produced by CD4<sup>+</sup>CD28<sup>-</sup> T cells in any condition, whereas IL-17A<sup>+</sup> and GM-CSF<sup>+</sup>/IL-17A<sup>+</sup> (double-positive) CD4<sup>+</sup>CD28<sup>+</sup> T cells were found in certain culture conditions. Interestingly, either anti-CD3 stimulation or IL-15 treatment alone resulted both in single- and double-positive CD4<sup>+</sup>CD28<sup>+</sup> T cells. In contrast, the combination of anti-CD3 stimulation and IL-15 did not result in IL-17A single-positive cells in any condition, but in all conditions, this cytokine was produced together with GM-CSF (Fig. 6B). In summary, these data reveal that CD4<sup>+</sup>CD28<sup>-</sup> T cells are not Th17 cells, but they are capable of producing significant amounts of GM-CSF upon challenge with IL-15.

#### IL-15 enhances the cytotoxic phenotype of CD4<sup>+</sup>CD28<sup>-</sup> T cells

We previously described the cytotoxic phenotype of CD4<sup>+</sup>CD28<sup>-</sup> T cells in MS patients (4, 9). In this study, we confirm that CD4<sup>+</sup>CD28<sup>-</sup> T cells express NKG2D, granzyme B, and perforin *ex vivo*. CD4<sup>+</sup>CD28<sup>+</sup> T cells did not express perforin, and only a small percentage of cells expressed granzyme B or NKG2D (Fig. 7A). No differences in *ex vivo* expression levels were found between HCs and patients ( $p > 0.05$ ). Next, we investigated the effect of IL-15 on the expression of cytotoxic molecules by CD4<sup>+</sup>CD28<sup>-</sup> T cells using flow cytometry. Treatment with IL-15 for 72 h significantly increased the expression of all cytotoxic molecules and of IFN-γ (Fig. 7B, 7C). The upregulation of IFN-γ was significantly higher in the patient population, compared with HCs ( $\Delta$ mean fluorescence intensity [MFI] 157.1 ± 9.5 versus MS:  $\Delta$ MFI 212.6 ± 15.4;  $p = 0.0084$ ). The upregulation of granzyme B and perforin was striking in both HC and MS patients, increasing 3- to 4-fold compared with the control condition (Fig. 7C). The upregulation of NKG2D was more subtle, increasing 2-fold compared with untreated cells (Fig. 7C). CD4<sup>+</sup>CD28<sup>+</sup> T cells did not upregulate NKG2D, and only small increases were seen for perforin, granzyme B, and IFN-γ ( $p > 0.05$ ). In summary, IL-15 enhances the cytotoxic phenotype of CD4<sup>+</sup>CD28<sup>-</sup> T cells in both HCs and MS patients.

#### IL-15 increases degranulation of CD4<sup>+</sup>CD28<sup>-</sup> T cells

As shown in this article, IL-15 increases the expression of cytotoxic molecules in CD4<sup>+</sup>CD28<sup>-</sup> T cells. However, these molecules have to be expelled before they have a cytolytic effect. Cytotoxicity can be accurately investigated using a flow-cytometric approach by



**FIGURE 5.** IL-15 induces proliferation of CD4<sup>+</sup>CD28<sup>-</sup> T cells. PBMCs of 10 HCs and 10 MS patients were stimulated with anti-CD3 (2 μg/ml), IL-2 (5 U/ml), IL-15 (10 ng/ml), anti-CD3+IL-2, anti-CD3+IL-15, or left unstimulated for 5 d. Flow cytometry was performed to identify proliferating (CFSE<sup>low</sup>) cells. (A) CD4<sup>+</sup> cells were gated; representative dot plots are shown. (B) The percentage of proliferated (CFSE<sup>low</sup>) CD4<sup>+</sup>CD28<sup>-</sup> T cells of HCs and MS patients is shown for each stimulation condition; dotted line represents unstimulated cells. Graphs represent mean values of all donors. ~ $p < 0.1$ , \* $p < 0.05$ , \*\*\* $p < 0.001$ .

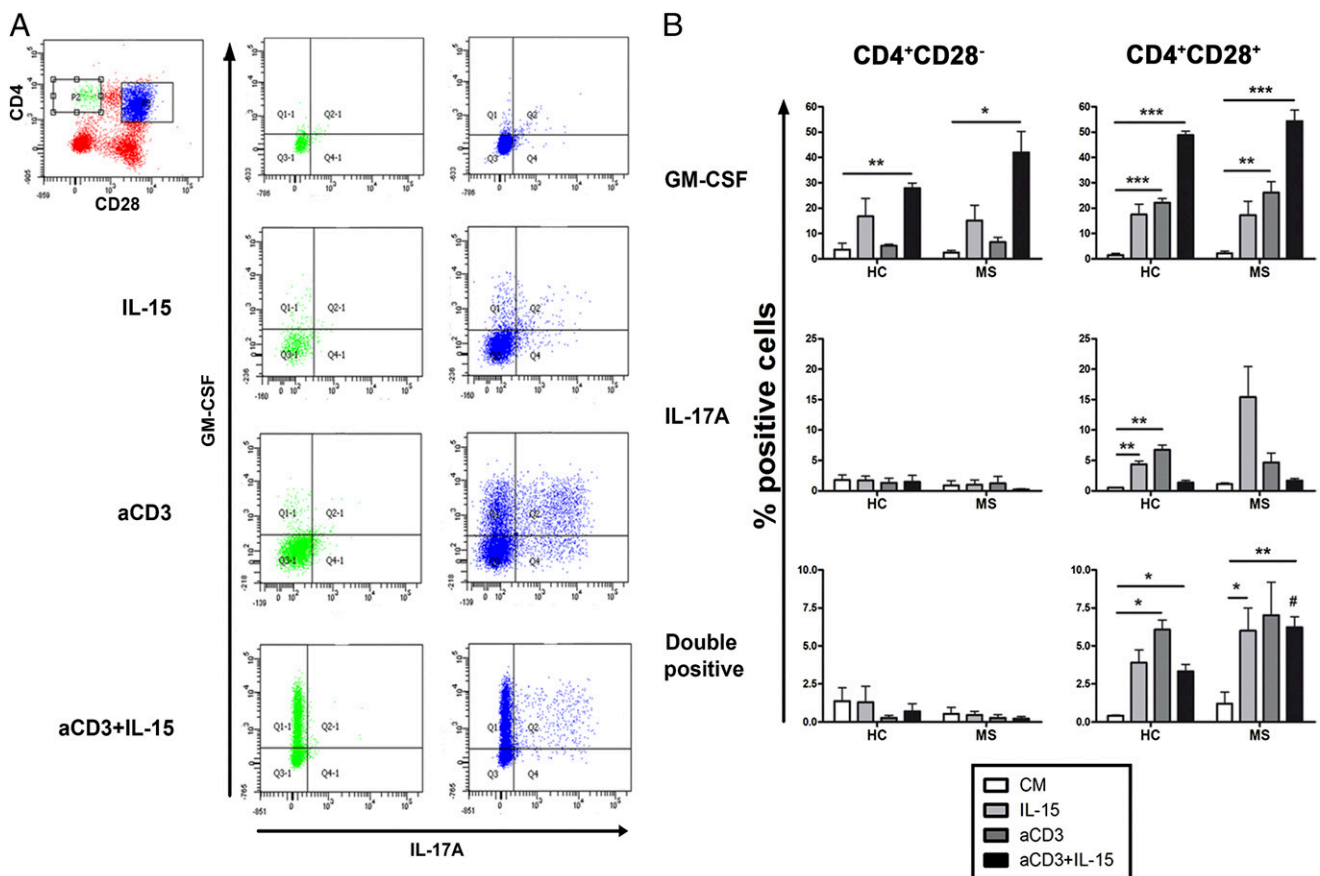
addressing the degranulation marker CD107a (43, 44). We investigated whether the degranulation of CD4<sup>+</sup>CD28<sup>−</sup> T cells is increased by IL-15. We treated cells with IL-15, and provoked degranulation by anti-CD3 stimulation, 4 h before flow-cytometric analysis. Already after 1 d of IL-15 treatment, degranulation was increased compared with anti-CD3 stimulation alone (only significant for HCs;  $p = 0.147$  for MS patients; Fig. 8B). At days 2 and 3, degranulation increased even further (Fig. 8A, 8B). There were no differences between HCs and patients; however, we could confirm the slightly higher ex vivo expression of CD107a in MS patients, which we reported in a previous study (4). To conclude, IL-15 increases the degranulation of CD4<sup>+</sup>CD28<sup>−</sup> T cells in both HCs and MS patients.

## Discussion

CD4<sup>+</sup>CD28<sup>−</sup> T cells arise from repeated antigenic stimulation and have a cytotoxic phenotype. They are capable of migrating to the CNS of MS patients via the fractalkine (CX<sub>3</sub>CL1–CX<sub>3</sub>CR1) system. These cells are present in a subpopulation of patients (45, 46) and have a cytotoxic phenotype, comparable with NK and CD8<sup>+</sup> T cells (4). IL-15, a cytokine that is increased in the cerebrospinal fluid and serum of MS patients (47), is known for its cytotoxicity-enhancing properties of cytolytic cells. In this study, we show that IL-15 is produced by astrocytes and infiltrating macrophages in MS lesions. Importantly, IL-15 selectively attracts CD4<sup>+</sup>CD28<sup>−</sup> T cells of patients and enhances transendothelial migration, possibly as a result of the increased expression of chemokine recep-

tors and adhesion molecules induced by IL-15. Further in vitro analyses indicated that IL-15 preferentially induces proliferation of CD4<sup>+</sup>CD28<sup>−</sup> T cells, induces production of GM-CSF, and increases expression of the cytotoxic molecules NKG2D, perforin, and granzyme B. Perforin and granzyme B are expelled after anti-CD3 stimulation, and this process is enhanced in the presence of IL-15. Taken together, our data indicate that upregulated IL-15 levels in MS patients strongly facilitate the migration of CD4<sup>+</sup>CD28<sup>−</sup> T cells across the blood–brain barrier. There, locally produced IL-15 (by astrocytes and infiltrating immune cells) further amplifies the pathogenic nature of these cells, thereby contributing to tissue damage in MS lesions.

In this study, we showed that increased IL-15 in MS patients has multiple effects on CD4<sup>+</sup>CD28<sup>−</sup> T cells, which are thought to contribute to the pathogenesis of MS. We found striking differences in the effect of IL-15 on either CD4<sup>+</sup>CD28<sup>−</sup> T cells or CD4<sup>+</sup>CD28<sup>+</sup> T cells. These differences are likely due to an increased activation of STAT5 (48) and an increased expression of IL-15R $\beta$ , which was reported on NKG2D<sup>+</sup>CD4<sup>+</sup> T cells (25). As our group has shown previously, NKG2D<sup>+</sup>CD4<sup>+</sup> T cells are mainly found in the CD28<sup>−</sup> subset (4). We found that IL-15 has a selective and potent chemotactic effect on CD4<sup>+</sup>CD28<sup>−</sup> T cells of MS patients, in addition to CX<sub>3</sub>CL1, previously reported to be a chemotactic component for CD4<sup>+</sup>CD28<sup>−</sup> T cells (4). Besides the chemotactic effect of IL-15 itself, we investigated whether IL-15 could promote migration through other chemokine axes. We found that IL-15 increased the expression of the chemokine receptor CX<sub>3</sub>CR1.

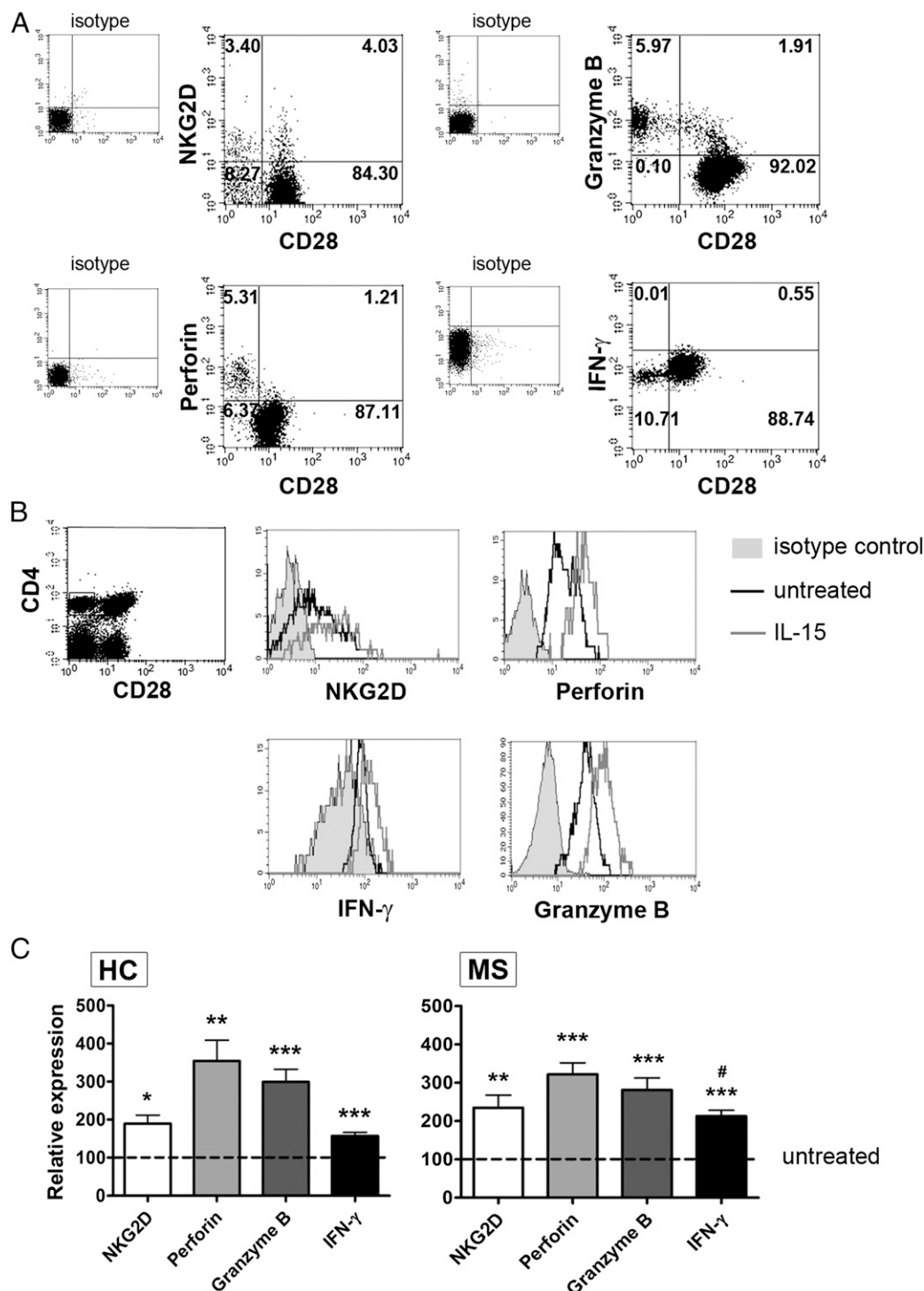


**FIGURE 6.** IL-15 induces production of GM-CSF, but not IL-17A, by CD4<sup>+</sup>CD28<sup>−</sup> T cells. PBMCs of three HCs and four MS patients were stimulated with 10 ng/ml IL-15, 2  $\mu$ g/ml anti-CD3, the combination of both, or left untreated. After 6 d, cells were stimulated with PMA/calcium ionomycin for 5 h and then stained intracellularly for IL-17A and GM-CSF. **(A)** Representative dot plots of one MS patient are shown for all culture conditions. **(B)** Within CD4<sup>+</sup>CD28<sup>−</sup> or CD4<sup>+</sup>CD28<sup>+</sup> cell populations, the percentage of cytokine-expressing cells is shown. Graphs represent mean values of all donors. CM, culture medium. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , # $p < 0.05$  between HCs and MS patients.



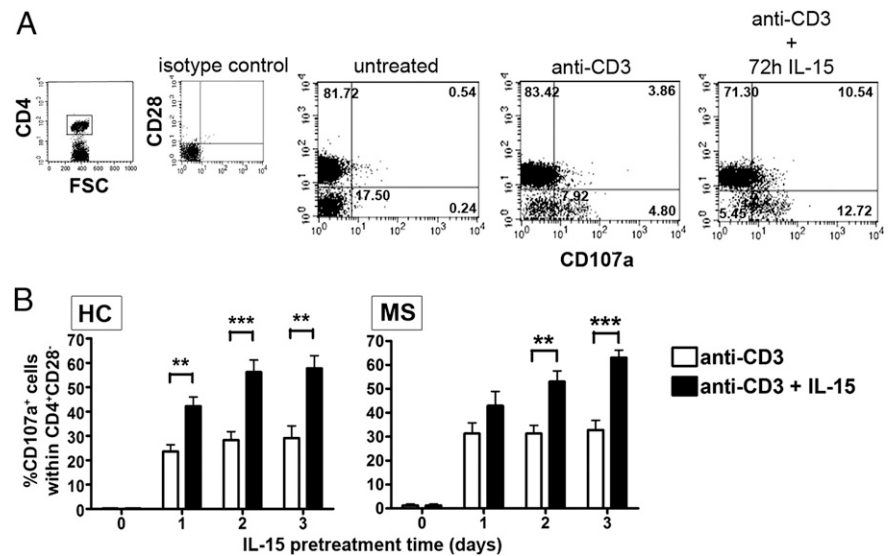
This is in contrast with the findings of Sechler et al. (49), who found that IL-15 decreased the expression of CX<sub>3</sub>CR1 on NK cells, and also the migration of these cells toward CX<sub>3</sub>CL1. This suggests that IL-15 has differential effects on different types of immune cells. In the case of CD4<sup>+</sup>CD28<sup>-</sup> T cells, we believe that IL-15 enhances their chemotactic capacity toward CX<sub>3</sub>CL1 and other chemokines, such as RANTES and MIP-1 $\alpha$  (CCR1 ligands),

which were found to be increased in MS lesions (50). In addition to upregulation of chemokine receptors, IL-15 also increased the expression of ICAM-1, LFA-1, and  $\alpha$ 4 integrin (molecules involved in transendothelial T cell migration) on CD4<sup>+</sup>CD28<sup>-</sup> T cells. In the case of  $\alpha$ 4 integrin, the increase was higher in MS patients compared with HCs, which could, in part, explain the elevated migration capacity of CD4<sup>+</sup>CD28<sup>-</sup> T cells of MS



**FIGURE 7.** IL-15 increases expression of cytotoxic molecules on CD4<sup>+</sup>CD28<sup>-</sup> T cells. PBMCs of 9 HCs and 12 MS patients were stimulated with IL-15 (10 ng/ml) or left unstimulated for 72 h. Flow cytometry was performed ex vivo [(A) gated on CD4<sup>+</sup> cells, representative dot plots from one healthy donor are shown] and after 72 h [(B) gated on CD4<sup>+</sup>CD28<sup>-</sup> cells] to determine the expression of NKG2D, perforin, granzyme B, and IFN- $\gamma$ . (C) The relative expression (MFI) of each marker (gated on CD4<sup>+</sup>CD28<sup>-</sup> cells) is shown compared with the untreated condition (dotted line). Graphs represent mean values of all donors. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , # $p < 0.01$  between HC and MS patients.

**FIGURE 8.** IL-15 increases degranulation of CD4<sup>+</sup>CD28<sup>−</sup> T cells. PBMCs of eight HCs and seven MS patients were stimulated with anti-CD3 (2  $\mu$ g/ml) with or without IL-15 (10 ng/ml) or left unstimulated for 3 d. Flow cytometry was performed ex vivo and every 24 h to determine the frequency of degranulating (CD107a<sup>+</sup>) cells. (A) CD4<sup>+</sup> cells are gated and representative dot plots are shown. (B) Percentages of degranulating (CD107a<sup>+</sup>) CD4<sup>+</sup>CD28<sup>−</sup> T cells are shown for HCs and MS patients. Graphs represent mean values of all donors. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.



patients. The higher susceptibility of MS-derived cells to IL-15 might be due to the increased transpresentation of IL-15 by APCs, as reported previously (24). Furthermore, we showed that IL-15 enhances the migratory potential of CD4<sup>+</sup>CD28<sup>−</sup> T cells across a monolayer of brain endothelial cells, confirming the functionality of IL-15-induced upregulation of adhesion molecules. Taken together, our data reveal that IL-15, upregulated in MS patients, enhances the migratory potential of CD4<sup>+</sup>CD28<sup>−</sup> T cells toward the CNS via induction of chemokine receptors and adhesion molecules.

When CD4<sup>+</sup>CD28<sup>−</sup> T cells enter the perivascular space, they encounter IL-15<sup>+</sup> myeloid cells. In vitro experiments revealed that IL-15 is not produced by T cells, and immunohistochemistry suggests that infiltrating macrophages or resident myeloid cells are the main IL-15 producers in this area. These data are confirmed by Saikali et al. (32), who also found IL-15 expression in perivascular infiltrates. The authors suggest that these monocytes present membrane-bound IL-15 to T cells, thereby leading to activation. This interaction has been described previously and is called *transpresentation* (22, 23). Transpresentation occurs when IL-15 is presented on the IL-15R $\alpha$  to cells expressing IL-2/IL-15R $\beta$  and the common  $\gamma$ -chain. This not only provides a signal to the receiving cell (leading to proliferation and activation), but also to the presenting cell via reverse signaling (leading to adhesion and cytokine production) (51). We suggest that IL-15 within the perivascular space activates both CD4<sup>+</sup>CD28<sup>−</sup> T cells (expressing the  $\beta$  and  $\gamma$  receptors) (24, 25) and monocytes/macrophages (expressing the  $\alpha$  receptor) (23), thereby contributing to the destructive immune responses in the brain. In addition to IL-15-expressing cells in the perivascular space, we also demonstrated that astrocytes express IL-15 in the brain parenchyma, confirming the findings of Saikali et al. (32). These data suggest that when CD4<sup>+</sup>CD28<sup>−</sup> T cells exit the perivascular space into the parenchyma (via an unknown mechanism), IL-15 can still have an effect on their function, adding to tissue damage in MS lesions.

Our in vitro results demonstrated that IL-15 affects CD4<sup>+</sup>CD28<sup>−</sup> T cells in multiple ways. We found that IL-15 induces proliferation of CD4<sup>+</sup>CD28<sup>−</sup> T cells, either alone or in combination with anti-CD3 stimulation. Its effect on the proliferation of T cells has long been recognized, since its discovery in 1994 (17). In this study, we demonstrated that IL-15 preferentially induced proliferation of CD4<sup>+</sup>CD28<sup>−</sup> T cells, compared with CD4<sup>+</sup>CD28<sup>+</sup> T cells of MS patients. These results are confirmed by the study of Alonso-Arias et al. (48), who analyzed the effect of IL-15 on

CD4<sup>+</sup>CD28<sup>−</sup> T cells of HCs. In addition, we showed that IL-15 could induce production of the encephalitogenic cytokine GM-CSF, but not IL-17, by CD4<sup>+</sup>CD28<sup>−</sup> T cells. The induction of GM-CSF in CD4<sup>+</sup>CD28<sup>−</sup> T cells nearly reached that in CD4<sup>+</sup>CD28<sup>+</sup> T cells, making them significant contributors to the total immune response in MS patients. Importantly, GM-CSF was recently found to be essential for EAE induction, whereas IL-17 expression was dispensable (42). Finally, the cytotoxic potential of CD4<sup>+</sup>CD28<sup>−</sup> T cells was enhanced by IL-15. This effect was previously described for CD8<sup>+</sup> T cells and NK cells (24, 52). In our study, expression of NKG2D, perforin, granzyme B, and IFN- $\gamma$  were increased, as well as the frequency of degranulating cells. In a previous study, we already showed that a large proportion of CD4<sup>+</sup>CD28<sup>−</sup> T cells degranulate in response to anti-CD3 stimulation. In addition, we found that the increased IFN- $\gamma$  production is more pronounced in MS patients compared with HCs.

Taken together, the presence of IL-15 may have consequences for the subgroup of MS patients who have an expanded population of CD4<sup>+</sup>CD28<sup>−</sup> T cells. Although studies correlating CD4<sup>+</sup>CD28<sup>−</sup> T cells to clinical phenotype are lacking, our study now suggests that either peripherally or locally increased levels of IL-15 may induce proliferation and activation of CD4<sup>+</sup>CD28<sup>−</sup> T cells, leading to a worsening of the immune response in MS lesions. Furthermore, our study demonstrates that IL-15 promotes the migratory and cytotoxic potential of CD4<sup>+</sup>CD28<sup>−</sup> T cells in MS patients. Adding to the complexity of a possible role of IL-15 in MS, two reports have demonstrated a beneficial effect of IL-15 on the course of EAE (53, 54). This can be attributed to the reliance of NK cells on this cytokine for their development, maturation, and function (18, 52). The role of NK cells in EAE and MS still remains controversial, because they can be either regulatory or detrimental (55). Therefore, targeting IL-15 as a new therapeutic strategy needs to be approached with caution; however, its effect on the pathogenicity of cytotoxic cells is worth exploring in more detail. Furthermore, future studies are needed to elucidate whether IL-15-induced phenotypic and/or functional changes in CD4<sup>+</sup>CD28<sup>−</sup> T cells can be blocked, to reduce the pathogenicity of this immune cell subset.

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## Disclosures

The authors have no financial conflicts of interest.

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