Increased expression of CD40 ligand (CD154) on CD4+ T cells as a marker of disease activity in rheumatoid arthritis

Beate Berner, Gabriele Wolf, Klaus M Hummel, Gerhard A Müller, Monika A Reuss-Borst

Abstract

Objectives—The interaction between the activation induced surface glycoprotein CD40L (ligand) (CD154) on CD4+ T cells and its receptor CD40, which is expressed on various cell types, plays a crucial part in numerous cell mediated and humoral immune reactions that may be of pathogenetic importance in rheumatoid arthritis (RA). To further evaluate the pathogenetic role of CD40L in RA, expression of CD40L and various other T cell activation antigens as well as costimulatory molecules was investigated on CD4+ T cells in RA by flow cytometry.

Methods—Two colour flow cytometry was used to determine the percentage of CD4+ T cells expressing CD40L, CD69, CD25, HLA-DR, CD39, CD27 and CD28 in peripheral blood (PB) of 62 RA patients in comparison to 20 healthy controls (HC). Disease activity was assessed by clinical, laboratory and radiological examination. Status of clinical remission of RA was evaluated according to the ACR preliminary criteria for complete clinical remission of RA.

Results—CD40L was expressed on > 10% of CD4+ T cells in 29% of RA patients thus defining a CD40L^{high+} patient group. Disease activity as estimated by C reactive protein, rheumatoid factor and status of clinical remission of disease ($p = 0.049$) was higher in this subgroup than in the RA CD40L^{low+} group. Expression of CD69, CD25, and HLA-DR was significantly increased in both RA patient groups in comparison with HC. However, the percentage of CD39+ CD4+ T cells was increased only in the RA CD40L^{high+} subgroup (versus HC $p = 0.019$, versus RA CD40L^{low+} $p = 0.044$). Furthermore, expression of CD40L and CD39 on CD4+ T cells correlated positively as estimated by Spearman rank correlation ($p < 0.001$). The percentage of CD4+ T cells lacking the costimulatory molecules CD27 ($p = 0.002$) and CD28 ($p = 0.026$) was increased in RA CD40L^{low+} patients in comparison with HC.

Conclusions—These data suggest that increased expression of CD40L on CD4+ T cells in RA indicates prolonged and increased activation of CD4+ T lymphocytes and is associated with active disease and possibly an unfavourable prognosis.

Whether this phenotypically defined RA CD40L^{high+} subgroup will preferentially respond to an anti-CD40L antibody treatment remains to be elucidated.

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The ability of T lymphocytes to transmit signals to various other cell types via distinct cell contact dependent mechanisms has been known for over a decade. CD40L (ligand) (CD154), a 33 kDa activation induced T cell surface glycoprotein, which is transiently expressed on activated, but not resting CD4+ T cells¹ is crucially involved in this cell-cell signalling process by binding to CD40. This phosphorylated glycoprotein belonging to the tumour necrosis factor receptor (TNFR) family^{2,3} is expressed on various cell types such as B cells, vascular endothelial cells, monocytes/macrophages, dendritic cells and fibroblasts. Thus, CD40L-CD40 interactions are involved in humoral and numerous cell mediated immune responses.^{3–5}

Ligation of activated CD40L+ CD4+ T cells to CD40 expressed on endothelial cells, for example, results in upregulation of certain adhesion molecules such as E-selectin (CD62E), VCAM-1 (CD106) or ICAM-1 (CD54) thus increasing leucocyte margination and diapedesis.⁴ Activation of histiocytes, dendritic cells and/or monocytes/macrophages by CD40L-CD40 signalling induces the production of chemokines and inflammatory cytokines as well as the synthesis of nitric oxide (NO) and metalloproteinases.⁴ Interaction of CD40L+ CD4+ T cells (that is, T helper cells) with CD40 on B cells causes B cell proliferation and differentiation, isotype switching and formation of B memory cells.⁵

The clinical importance of CD40L expression in vivo is further highlighted by studies of the hyper-IgM-syndrome. This human X-linked immunodeficiency characterised by absent or low levels of IgG, IgA and IgE in serum, but normal or increased levels of IgM and defects in T cell mediated immunity is caused by mutations in the CD40L gene that result in a lack of functional expression of CD40L on activated T cells.⁶

In patients with systemic lupus erythematosus (SLE) baseline expression of CD40L and CD40L regulation was recently shown to be changed.^{7,8} Furthermore, anti-CD40L antibody treatment in mice produced longstanding disease remissions stressing the pathogenetic

Department of Nephrology and Rheumatology, University Medical Clinic, Robert-Koch-Str 40, 37075 Göttingen, Germany

Correspondence to: Dr Berner

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importance of CD40L-CD40 interactions in murine SLE.⁹⁻¹¹

With regard to rheumatoid arthritis (RA), the most frequent autoimmune rheumatic disease characterised by chronic inflammation and proliferation of the synovium and consecutive cartilage and bone destruction, the pathogenetic importance of CD40L-CD40 interactions remains to be elucidated, in particular as the contribution of T cells to RA has been a matter of debate for years. Several lines of evidence, however, support the hypothesis of a T cell driven disease such as the observation that T cells are the dominant cell population in the synovial infiltrate, the association with certain MHC class II molecules and at least the partial therapeutic effect of T cell depletion.¹² In this study, we provide evidence that the activation induced T cell antigen CD40L may finally prove to be such an important marker of disease activity and possibly unfavourable prognosis. Therefore, the expression of CD40L by CD4+ T cells was investigated in 62 peripheral blood (PB) and 10 synovial fluid (SF) samples of patients with RA in comparison with 20 healthy controls (HC) and results were correlated with clinical and laboratory disease status.

Methods

Heparinised PB samples of 62 patients with RA and 20 HC were investigated. All patients fulfilled the ARA revised criteria 1987.¹³ Patients (51 women and 11 men) ranged in age from 25 to 77 years (mean age 58.4). By clinical and radiological evaluation patients were categorised as having early disease (that is, no radiological evidence of bone erosions, no extra-articular features) (10 patients), progressive disease (that is, continuous disease activity, radiological evidence of bone erosions, possibly extraarticular features) (51 patients) and late disease (that is, disease duration of many years, residual joint damage) (one patient). Extra-articular features were present in nine patients (four with rheumatoid nodules, four with a sicca syndrome and one with a vasculitis). Disease activity of RA was assessed by level of C reactive protein (CRP). Sixty six per cent of RA

patients received disease modifying antirheumatic drugs (DMARD). Status of clinical remission of disease was determined according to the ACR preliminary criteria for complete clinical remission of RA.¹⁴ Table 1 gives the patient characteristics. Additionally, SF was obtained from 10 patients with RA by arthrocentesis.

DIRECT IMMUNOFLOUORESCENCE AND FACS ANALYSIS

Immunophenotypical analysis was performed using a large panel of directly labelled monoclonal antibodies against various lymphoid differentiation and activation antigens. Antibodies against CD27 (clone M-T271), CD28 (clone CD28.2), CD39 (clone Tü 66) and CD40L (clone TRAP 1) were purchased from Pharmingen (San Diego, CA, USA), antibodies against CD25 (clone B1.49.9) were obtained from Coulter-Immunotech Diagnostics (Hamburg, Germany). Antibodies against CD3 (Leu 4), CD4 (Leu 3a), CD69 (Leu 23) as well as $\gamma 1/\gamma 2$ -controls were purchased from Becton Dickinson (San José, CA, USA). Results of CD40L expression were reproduced by using another two monoclonal antibodies against CD40L, clone 89-76 (Becton Dickinson, San José, CA, USA) and clone 24-31 (Ancell Corporation, Bayport, MN, USA).

Briefly, 20 μ l fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibody was given to 200 μ l heparinised PB and incubated for 15 minutes. Two ml FACS-Lysing-Solution (Becton Dickinson, San José, CA, USA) was added and incubated for 15 minutes. Cells were then washed twice and resuspended in phosphate buffered saline (PBS) (Biochrom KG, Berlin, Germany) for flow cytometry. SF was drawn aseptically into heparinised tubes. SF mononuclear cells were isolated by Ficoll (Biochrom, Berlin, Germany) gradient centrifugation. Then $2 \times 10^5 - 1 \times 10^6$ cells in 200 μ l PBS were incubated with 20 μ l FITC or PE labelled monoclonal antibody for 15 minutes. Cells were washed twice and resuspended in PBS for analysis.

For two colour analysis PB and SF cells were analysed on a FACS-Calibur (Becton Dickinson, San José, CA, USA) using cellquest software (Becton Dickinson, San José, CA, USA). Data of 10 000 cells/sample were collected for forward light scatter (FSC) and sideward light scatter (SSC) in linear scale, for fluorescein (530 nm band pass (bp)) and phycoerythrin (580 nm bp) fluorescence in log scale. Analysis was done by gating on mononuclear cells in FSC/SSC dot plots. Non-specific immunofluorescence was determined by using negative control antibodies and subtracted from specific immunofluorescence. The percentage of FITC and PE positive cells and the mean fluorescence levels were calculated.

IMMUNOHISTOCHEMISTRY

Six μ m frozen cryostat sections of synovial tissue (ST) of a RA patient were analysed for CD40L expression by direct immunofluorescence. Sections were air dried and fixed in

Table 1 Patient characteristics

	RA CD40L ^{high+} (n=18)	RA CD40L ^{low+} (n=44)
Mean disease duration (range) in years	7 (0.5-25)	7 (0.5-40)
Mean age (range) in years	54.6 (29-75)	61.5 (25-77)
Male/female	4 / 14	7 / 37
State of disease		
early / progressive / late	3 / 15 / 0	7 / 36 / 1
Extraarticular features		
nodules / sicca syndrome / vasculitis	1 / 2 / 0	3 / 2 / 1
Increased CRP level	15 (83%)	28 (64%)
Mean (SD) CRP (mg/l)	30.2 (24.1)	36.5 (34.7)
Positive RF	16 (89%)	32 (73%)
Mean (SD) RF (IU/ml)	292.8 (306.3)	240.5 (453.1)
Complete Remission	0 (0%)	9 (21%)
DMARD medication		
methotrexate	10	20
sulfasalazine	2	8
hydroxychloroquine	0	2
cyclosporin A	3	0
no DMARD	6 (33%)	15 (34%)

Characteristics of RA CD40L^{high+} (n=18) and RA CD40L^{low+} (n=44) patients. SD: standard deviation, CRP: C reactive protein, RF: rheumatoid factor, DMARD: disease modifying antirheumatic drug.

Figure 1 Percentage of PB CD4+ T cells expressing CD40L in healthy controls (HC, $n = 20$), RA CD40L^{low+} ($n = 44$) and RA CD40L^{high+} ($n = 18$) patients as well as percentage of SF CD4+ CD40L+ T cells in RA patients (SF, $n = 10$). The bars indicate the medians.

acetone for five minutes. Then they were rehydrated with TRIS buffered saline (TBS) for five minutes, blocked for 60 minutes in 5% swine serum (DAKO Corporation, Carpinteria, CA, USA) in TBS and incubated with a PE labelled anti-CD40L antibody (clone 24-31, Ancell Corporation, Bayport, MN, USA) at 4°C for 12 hours. Finally, ST sections were counterstained with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim GmbH, Mannheim, Germany) for 15 minutes. Imaging was done using fluorescence microscopy (Axiovert S 100 TV, Carl Zeiss GmbH, Jena, Germany). With the exception of incubation with the primary antibody all incubations were done at room

temperature. Sections were washed with TBS after each incubation, and primary antibody was diluted in TBS. An irrelevant isotype matched PE labelled antibody (Coulter-Immunotech Diagnostics, Hamburg, Germany) was used for control staining.

STATISTICS

Statistical analysis was performed with Sigma-Stat (Jandel Scientific). Median as well as 25% and 75% percentile of data were calculated. Statistical significance between groups was determined by Mann-Whitney rank sum test. Results were considered to be statistically

Figure 2 Fluorescence activated cell sorter histograms of CD40L expression (gating on CD4+ T cells) demonstrating the intensity and density of CD40L antigen expression patterns (thin line) defined as CD40L^{high+} (A) and CD40L^{low+} (B) expression. The negative control was obtained by using a isotype monoclonal antibody (thick line).

Figure 3 Intraindividual variability of CD40L expression in 8/18 RA CD40L^{high+} patients. Under intensified immunosuppressive treatment the percentage of CD40L+ CD4+ T cells declined in seven of eight patients, but remained in seven of eight patients > 10%. The mean time (t1-t2) was eight months (ranging from 3 to 20 months).

Table 2 Expression of activation and costimulatory molecules on PB CD40L^{high+} and CD40L^{low+} CD4+ T cells in RA

	HC (n=20)	RA CD40L ^{high+} (n=18)	RA CD40L ^{low+} (n=44)	RA CD40L ^{high+} versus HC	RA CD40L ^{low+} versus HC	RA CD40L ^{high+} versus RA CD40L ^{low+}
Activation antigens						
CD69 (%)	4.0 (2.0;7.75)	17.0 (7.0;25.25)	14.0 (8.25;22.5)	p<0.001	p<0.001	NS
CD25 (%)	13.0 (9.5;16.0)	19.0 (12.75;22.25)	19.0 (13.0;21.0)	p=0.042	p=0.009	NS
HLA-DR (%)	0.0 (0.0;2.0)	7.0 (1.5;17.0)	2.0 (0.0;7.25)	p=0.009	p=0.032	NS
CD39 (%)	5.0 (3.0;6.0)	10.0 (5.0;27.0)	5.0 (3.0;8.0)	p=0.019	NS	p=0.044
Costimulatory molecules						
CD27 (%)	93.5 (88.0;96.0)	83.5 (69.0;97.0)	83.0 (75.0;93.0)	NS	p=0.002	NS
CD27- (%)	6.5 (4.0;12.0)	16.5 (3.0;31.0)	17.0 (7.0;28.0)	NS	p=0.002	NS
CD28 (%)	98.0 (96.0;100.0)	98.0 (91.0;100.0)	96.0 (93.0;98.0)	NS	p=0.026	NS
CD28- (%)	2.0 (0.0;4.0)	2.0 (0.0;9.0)	4.0 (2.0;7.0)	NS	p=0.026	NS

Percentage of PB CD4+ T cells expressing the activation antigens CD69, CD25, HLA-DR and the costimulatory molecules CD27 and CD28 are shown. The percentage of PB CD4+ T cells lacking the expression of CD28 (CD28-) and CD27 (CD27-) are listed, too. Medians and 25% and 75% percentile ranks (in parentheses) are given. Statistical significance between groups (healthy controls (HC), RA CD40L^{high+} and RA CD40L^{low+} patients) was determined by Mann-Whitney rank sum test. p Values >0.05 were considered to be not statistically significant (NS).

significant at a p value < 0.05. Correlation was estimated by Spearman rank correlation. Correlation was considered at a p value < 0.05.

Results

Expression of CD40L on PB CD4+ T cells was evaluated in 62 RA patients and 20 HC by two colour flow cytometry. CD40L expression was significantly increased in RA patients (median 6.5%) in comparison with HC (median 2.5%, p < 0.001). In addition, CD40L was strongly expressed on > 10% of CD4+ T cells in 18 of 62 (29%) of RA patients but 0 of 20 (0%) of HC (fig 1). These data were obtained by using the anti-CD40L-antibody TRAP1 and could be confirmed by using two other antibodies directed against CD40L (clone 89-76 and 24-31). According to the percentage and density of antigen expression of CD40L two groups of RA patients could be determined: patients with ≤ 10% CD40L+ CD4+ T cells (RA CD40L^{low+}) and patients with > 10% CD40L+ CD4+ T cells (RA CD40L^{high+}) (fig 2). Table 1 shows the clinical characteristics of both patient groups. There were no differences between these groups with regard to disease duration, age of patients, male/female ratio, state of disease, occurrence of extra-articular features and medication. Remarkably, 0 of 18 (0%) patients were in clinical remission in the RA CD40L^{high+} subgroup, whereas 9 of 44 (21%) CD40L^{low+} patients fulfilled the ACR

preliminary criteria for complete clinical remission¹⁴ at the time of analysis (p = 0.049). The percentage of patients with an increased CRP level was higher in the CD40L^{high+} subgroup (83%) than CD40L^{low+} patients (64%). In addition, a positive RF was more often observed in CD40L^{high+} (89%) than CD40L^{low+} (73%) patients. Eight of 18 CD40L^{high+} patients and 8 of 44 CD40L^{low+} patients were reanalysed several months later. The percentage of CD40L+ CD4+ T cells remained > 10% in seven of eight CD40L^{high+} patients showing, however, a tendency to decline under intensified immunosuppressive treatment—that is, increased doses of prednisone in all patients and initiation of a new/additional DMARD medication (sulfasalazine, hydroxychloroquine and cyclosporin A) in three patients (fig 3). These patients did not achieve clinical remission and inflammatory laboratory markers remained increased. Within the CD40L^{low+} subgroup, expression of CD40L on CD4+ T cells did not exceed 10% on repeated analysis. Clinically, these patients had stable disease. SF was investigated in 10 RA patients with CD40L being expressed on a significantly higher percentage of SF than PB CD4+ T cells in CD40L^{low+} patients (p < 0.001). Of the CD40L^{high+} patient group, two matched samples of PB and SF were obtained for analysis. Remarkably, in both patients CD40L was expressed on a higher percentage of PB than SF CD4+ T cells. In ST obtained from one of these two CD40L^{high+} patients, immunofluorescence microscopy revealed CD40L expression on infiltrating mononuclear cells.

Coexpression of the activation antigens CD69, CD25, HLA-DR and CD39 as well as the costimulatory molecules CD27 and CD28 was studied on CD4+ T cells. Results are summarised in table 2. The expression of CD69, CD25 and HLA-DR was significantly increased in the CD40L^{high+} and CD40L^{low+} subgroup when compared with HC. Between the two RA subgroups no differences in the expression of these activation antigens could be detected. In contrast, the expression of the activation antigen CD39 was significantly increased in the CD40L^{high+} group (p = 0.044) in comparison with the CD40L^{low+} patients. Moreover, expression of CD40L and CD39 on

Figure 4 The percentage of CD39+ CD4+ T cells correlated significantly (p < 0.001) with the expression of CD40L in the RA CD40L^{high+} group as determined by Spearman rank correlation. Data are shown in logarithmic scale.

CD4⁺ T cells correlated positively in CD40L^{high+} patients as could be shown by Spearman rank correlation (correlation coefficient 0.765, $p < 0.001$) (fig 4). No difference in the expression of CD39 could be observed between CD40L^{low+} patients and HC. Expression of the costimulatory molecules CD27 and CD28 showed similar results in both RA subgroups. The percentage of T cells lacking the expression of CD27 and CD28 was increased in RA patients in comparison with HC. Statistically significant differences in antigen expression could, however, only be detected between CD40L^{low+} patients and HC (CD27: $p = 0.002$; CD28: $p = 0.026$).

Discussion

The signalling between the TNFR CD40 and its ligand CD40L plays a crucial part in the immune system contributing to cell mediated as well as humoral immune responses.³⁻⁵ It is known that CD40L forms a homotrimeric complex on the surface of activated T cells, to which three CD40 molecules can bind by fitting into the interface between adjacent CD40L monomers.^{15, 16} CD40 receptor binding to CD40L trimer induces clustering of the receptors thus initiating signal transduction.^{2, 3}

In RA, the chronic tissue destructive process has been attributed to an ongoing antigen driven immune response in which activated T cells play an important inflammatory part.¹² Numerous CD40L-CD40 mediated inflammatory reactions such as induction of proinflammatory cytokines and NO production as well as upregulation of costimulatory activity of dendritic cells and monocytes/macrophages^{3, 4} are known to be of functional and pathogenetic relevance in this destructive joint disease. In particular, CD40-CD40L interaction regulates IL12 production of dendritic cells, which is required for induction of Th1 type responses.⁴ As RA has been identified as a Th1 cell type mediated disease, this pathogenetic pathway may be another important function of CD40L-CD40 interaction.¹⁷ Furthermore, CD40L⁺ T cells infiltrating the joints in RA could interact with CD40⁺ synovial fibroblasts causing their proliferation and upregulation of CD54 (ICAM-1),¹⁸ which could result in further recruitment of inflammatory cells in the synovium, as well as increasing the production of tumour necrosis factor α , the key inflammatory cytokine in RA.¹⁹

In a recent study, MacDonald *et al*²⁰ showed expression of CD40L mRNA by PB and SF T cells from RA patients, whereas CD40L cell surface expression was only observed on a small percentage of PB and SF T cells. In contrast with this study we provide evidence that CD40L is strongly expressed on CD4⁺ T cells in a particular subset of RA patients (29%) thus phenotypically defining a CD40L^{high+} RA subgroup. CD40L expression was significantly higher in SF than PB of CD40L^{low+} RA patients; however, simultaneous analysis of PB and SF in 2 of 18 RA CD40L^{high+} patients revealed a higher percentage of CD40L⁺ CD4⁺ T cells in PB than SF. In one of these two CD40L^{high+} patients CD40L expression

was, however, documented in ST. Hence, one might speculate that CD40L⁺ CD4⁺ T cells preferentially migrate from PB into the synovial tissue, where they may interact with CD40 being expressed on different cell types in particular on synovial fibroblasts.^{19, 20}

In addition, expression of several activation antigens and costimulatory molecules was investigated on CD4⁺ T cells. In accordance with previous studies,²¹⁻²⁴ the expression of very early, early and late activation antigens as CD69, CD25 and HLA-DR on PB CD4⁺ T cells of RA patients was significantly increased in the RA CD40L^{high+} and RA CD40L^{low+} subgroups compared with HC. There were no differences in the expression of these antigens between RA CD40L^{high+} and RA CD40L^{low+} patients and expression of these antigens could not be correlated with CD40L expression. Interestingly, expression of CD39, a lymphoid activation marker with prolonged expression after activation,²⁵ mediating homotypic adhesion^{25, 26} and showing ecto-apyrase activity,²⁷ was significantly increased and positively correlated with CD40L expression in the RA CD40L^{high+} group. Thus, CD40L⁺CD39⁺ CD4⁺ T cells probably constitute a distinct subset of CD4⁺ T lymphocytes that have undergone prolonged and increased activation. On repeated analysis, expression of CD40L showed intraindividual variability. This observation strongly argues against a genetic control of CD40L expression in the RA CD40L^{high+} subgroup and favours the hypothesis of different lymphocyte activation levels at distinct phases of disease.

In agreement with recent results²⁸⁻³⁰ CD4⁺ T cells lacking expression of the costimulatory molecule CD28 were augmented in the investigated RA cohort. Remarkably, an increased percentage of CD28⁻ CD4⁺ T cells was only found within the RA CD40L^{low+} subgroup. Similarly, the percentage of CD4⁺ T cells lacking the costimulatory molecule CD27, another member of the TNFR family being expressed on discrete subpopulations of T and B cells and providing costimulatory signals for B and T cell proliferation,³¹ was increased in the RA CD40L^{low+} subset. This is in accordance with a previous report by Kohem *et al*.³²

So far, it seems that strong expression of CD40L on CD4⁺ T cells reflects augmented and prolonged activation of lymphocytes. Remarkably, CD40L expression correlated with increased disease activity as the percentage of patients with increased CRP level and positive RF was higher in the RA CD40L^{high+} than in the RA CD40L^{low+} subgroup. Furthermore, the percentage of RA CD40L^{high+} patients (0%) in clinical remission of disease was significantly lower than that of CD40L^{low+} patients (21%). Thus, the expression of CD40L is associated with active disease and possibly an unfavourable prognosis. This probably may be attributable to chronic and prolonged activation of T lymphocytes and consecutively raised and prolonged inflammatory activity of disease. Interestingly, expression of CD40L was not restricted to very early

stages of disease, but also observed after 5–12 years disease duration.

The therapeutic significance of our observations remains to be investigated. In SLE, encouraging results have been obtained in mice with an anti-CD40L antibody treatment. This applies in particular to the onset of renal disease, which could be delayed by anti-CD40L-antibody treatment.¹⁰ Preliminary experiments have also reported a beneficial effect of anti-CD40L antibody treatment in collagen induced arthritis of mice when applied at the time of immunisation.³³ As pointed out above, numerous cell-cell interactions that are essential for the chronic disease process in RA could theoretically be disrupted by giving anti-CD40L antibody. The finding that CD40L is hyperexpressed in a subset of RA patients may thus have implications for evaluating this antibody in human RA and anti-CD40L antibody treatment may finally prove to be a novel, rather specific immunotherapeutic approach in this particular subgroup of patients.

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