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Research paper

Isolation of vascular smooth muscle antigen-reactive CD4$^+$αβTh1 clones that induce pulmonary vasculitis in MRL/Mp-*Fas*$^{+/+}$ mice

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ABSTRACT

Here, we established CD4$^+$αβTh1 clones specific for rat vascular smooth muscle antigen (VSMAg) that induced vasculitis lesions in the lungs of MRL/Mp-*Fas*$^{+/+}$ mice following adoptive transfer. Six different T cell clones, MV1b1 (Vβ1), MV1b4 (Vβ4), MV1b8.3 (Vβ8.3), MV1b61 (Vβ6), MV1b62 (Vβ6), and MV1b63 (Vβ6), were isolated from the MV1 T cell line from the regional lymph nodes of immunized MRL/Mp-*Fas*$^{+/+}$ mice; the three (Vβ6) clones had unique CDR3 amino acid sequences. Following stimulation with VSMAg-pulsed antigen presenting cells, MV1b61 and MV1b62 failed to secrete interferon-γ and tumor necrosis factor-α, although the other four clones secreted high levels of both cytokines. In adoptive transfer experiments, MV1b61 and MV1b62 did not induce organ involvement including pulmonary vasculitis. In contrast, MV1b1, MV1b4, MV1b8.3, and MV1b63 induced perivascular mononuclear cell infiltration in pulmonary small arteries. These clones may provide useful tools for investigating the underlying mechanisms of vasculitis syndromes and for developing therapeutic strategies.

1. Introduction

The pathogenesis of vasculitis syndromes is thought to be associated with the autoimmune process. The underlying mechanism of the reported vasculitis syndrome animal models is thought to be the deposition of immune complexes in blood vessels [1]. However, several human vasculitis syndromes such as granulomatosis with polyangiitis, microscopic polyangiitis, and eosinophilic granulomatosis with polyangiitis do not exhibit immune complex deposition [2]. Recently, the presence of disease-specific anti-neutrophil cytoplasmic antibody (ANCA) has been demonstrated for certain types of vasculitis, termed ANCA-associated vasculitis. In addition, T cells are known to play an important role in the development of several vasculitis syndromes [3]. Specific changes in the CD4$^+$T cell receptor Vβ repertoire have been demonstrated in primary systemic vasculitis in childhood, such as Henoch-Schonlein purpura and Kawasaki disease [4]. The T cells infiltrating the walls of coronary arterial aneurysms and intestinal mucosa of Kawasaki disease patients exhibit a skewed T cell receptor (TCR) Vβ profile, with increased numbers of cells expressing Vβ2 [5,6]. Moreover, abnormal expansions and deletions of T cells with a particular Vβ have been found in the peripheral blood of adults with granulomatosis with polyangiitis [7], Takayasu arteritis [8], giant cell arteritis [9], and eosinophilic granulomatosis with polyangiitis [10].

Murine models of vasculitis have been previously reported. Spontaneous vasculitis with CD4$^+$T cell infiltration of the vessel walls was identified in MRL/Mp-*Fas*^{lpr} mice [11]. Hart et al. reported that mouse lymphocytes cultured with syngeneic vascular smooth muscle cells induce vasculitis following adoptive transfer [12]. Based on these observations, we hypothesized that vascular component-specific CD4$^+$T cells could cause vasculitis in susceptible animals.

Previously, we established a CD4$^+$T cell line, MV1, from the regional lymph nodes of MRL/Mp-*Fas*$^{+/+}$ mice, which proliferates in the presence of vascular smooth muscle antigen (VSMAg)-pulsed antigen presenting cells (APC) [13]. The MV1 line induces pulmonary vasculitis in MRL/Mp-*Fas*$^{+/+}$ mice by adoptive transfer and possesses cytotoxic activity, mediated by the Fas-Fas ligand pathway [13]. We therefore hypothesized that specific T cell clones of the MV1 line could play an important role in the pathogenesis of vasculitis. In the present study, T cell clones involved in pulmonary vasculitis in murine lupus were isolated from the MV1 line. In addition, the expression of TCR Vβ, the complementary determin-

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ing region 3 (CDR3) sequence, and cytokine secretion in the isolated T cell clones were analyzed to further elucidate the role of T cells in the pathogenesis of vasculitis.

2. Materials and methods

2.1. Animals

Female MRL/Mp-*Fas*$^{+/+}$ mice (aged 8–12 weeks) were purchased from SLC (Shizuoka, Japan). Mice were maintained in a specific pathogen-free environment at Kanazawa Medical University. All procedures were approved by The Kanazawa Medical University Animal Welfare Committee.

2.2. Cell culturing

The MV1 T cell line was cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol, at 37 °C, under 5% CO_2. Irradiated (30 Gy) syngeneic spleen cells (2×10^6/mL) were added weekly, together with VSMAg (1×10^5 cells/mL). The MV1 T cell line was transferred at a limiting dilution to 96-well plates at a concentration of 0.5 cells/well. Growing cells (<5/plate) were then harvested and further expanded in 24-well plates.

2.3. Flow cytometry

Isolated T cell clones were analyzed by FACSCalibur® (Becton-Dickinson, Franklin Lakes, NJ, USA) using fluorescein isothiocyanate (FITC)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD8, FITC-conjugated anti-CD3, and FITC-conjugated anti-T cell receptor Vβ antibodies. Isolated T cell clones were examined to determine the type of T cell receptor Vβ chain contained in the cells. All antibodies were purchased from Becton-Dickinson.

2.4. Amino acid sequencing of the TCR Vβ CDR3 region

Total RNA was extracted from isolated T cell clones with the TRIzol® reagent (GIBCO/BRL, Carlsbad, CA, USA). Five micrograms of total RNA were subjected to reverse-transcription PCR (RT-PCR) using specific primers that recognized the CDR3 region of each TCR Vβ. PCR products were electrophoresed on a 1.5% agarose gel and the predicted-size single bands were detected; these bands were gel purified using the GENECLEAN kit (Funakoshi, Tokyo, Japan) and then subjected to direct sequencing.

2.5. Proliferation of T cell clones

T cell clones (1×10^4 cells) were cultured in flat-bottomed 96-well plates for 72 h with VSMAg, irradiated (30 Gy) syngeneic spleen cells (5×10^5 cells/well), and antigen presenting cells (APC) in triplicate. During the final 18 h, 37 kBq of methyl-^{3}H-thymidine was added to each well and cells were harvested with a semi-automatic cell harvester (Skatron Instruments, Sterling, VA, USA). Scintillation counting of incorporated ^{3}H-thymidine (count/min) served as the measure of T cell proliferation.

2.6. Cytokine secretion by T cell clones

T cell clones (1×10^4 cells) were stimulated with plate-fixed anti-CD3 antibody or co-cultured with irradiated (30 Gy) syngeneic spleen cells (5×10^5 cells/well) in the presence or absence of VSMAg in flat-bottomed 96-well plates for 48 h. The levels of cytokines interferon-γ (IFN-γ), interleukin-4 (IL-4), and tumor necrosis factor-α (TNFα) in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA).

2.7. Adoptive transfer of T cell clones

T cell clones were stimulated with VSMAg (1×10^5 cells/mL) for 4 days in the presence of irradiated (30 Gy) syngeneic spleen cells. To suppress regulatory T cells, recipient mice were pretreated 4 days prior to the first T cell transfer with an intraperitoneal injection of 20 mg/kg cyclophosphamide. The stimulated T cell clones (1×10^7 cells/mouse/per transfer) were then transferred twice into MRL/Mp-*Fas*$^{+/+}$ mice by intraperitoneal injection; the first transfer was performed 4 days subsequent to the cyclophosphamide pretreatment and the second transfer took place 4 days following the first transfer. Four days following the second transfer, the mice were sacrificed, and the lung, heart, kidney, and aorta were fixed in 10% buffered formaldehyde and stained with hematoxylin and eosin. The histology of the pulmonary vasculitis was scored as described in Fig. 1; the histopathology scores presented are the sum of four experiments.

3. Results

3.1. MV1 T cell line cloning by limiting dilution

As described in the Section 2, MV1 T cell line clones were isolated by limiting dilution. Twenty-four T cell lines were generated, of which 14 were determined to be the most highly VSMAg-specific, based on proliferation assays. Briefly, T cells were co-cultured with APC with or without VSMAg (1×10^5 cells/mL); selected lines exhibited simulation indices > 4, counts per minute with stimulation > 20,000, and counts per minute without stimulation < 1000. The derived T cell lines were CD4$^+$, CD8$^-$, CD3$^+$, and TCRαβ$^+$, as demonstrated by flow cytometry; the cells lines were thought to be clones since they shared a single TCR Vβ repertoire except for one line. The surface phenotype of the T cell clones was consistent with that observed in our previous study demonstrating that the MV1 T cell line is CD4$^+$, CD8$^-$, CD3$^+$, and TCRαβ$^+$.

Using specific primers, the DNA sequences of the TCR Vβ CDR3 regions were amplified by RT-PCR and subjected to direct sequencing. A number of clones shared the same CDR3 sequence, presumably since they were clonal replicates. Therefore, we derived six different clones from MV1 (Table 1) named MV1b1 (Vβ1), MV1b4 (Vβ4), MV1b8.3 (Vβ8.3), MV1b61 (Vβ6), MV1b62 (Vβ6), and MV1b63 (Vβ6). The three clones that possessed TCR Vβ6 had different CDR3 amino acid sequences; these sequences were not characteristic TCR Vβs in terms of charge, hydrophilicity, or the length of the CDR3 region. Vβ6 was the most frequently identified sequence that could be associated with vasculitis pathogenesis in our model. The proliferation assay results for the six clones isolated in the presence of VSMAg are shown in Table 2.

3.2. Cytokine secretion by T cell clones

Cytokine secretion upon stimulation with anti-CD3 antibody (data not shown) and VSMAg (1×10^5 cells/mL; Fig. 1) were measured by ELISA. All clones produced IFNγ (Fig. 2A) but none secreted IL-4 (data not shown), which suggests the clones are Th1 cells. TNFα was also secreted in all clones (Fig. 2B), mostly correlating with IFNγ production. In contrast, cytokines were not secreted in any of the clones in the absence of VSMAg. We hypothesized that the clones were stimulated specifically by VSMAg and that the level of cytokine secretion might be associated with the