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Environmental factor and inflammation-driven alteration of the total peripheral T-cell compartment in granulomatosis with polyangiitis

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ABSTRACT

Autoimmune diseases are initiated by a combination of predisposing genetic and environmental factors resulting in self-perpetuating chronic inflammation and tissue damage. Autoantibody production and an imbalance of effector and regulatory T-cells are hallmarks of autoimmune dysregulation. While expansion of circulating effector memory T-cells is linked to disease pathogenesis and progression, the causes driving alterations of the peripheral T-cell compartment have remained poorly understood so far. In granulomatosis with polyangiitis (GPA), a prototypical autoimmune disorder of unknown aetiology, we performed for the first time a combined approach using phenotyping, transcriptome and functional analyses of T-cell populations to evaluate triggers of memory T-cell expansion. In more detail, we found increased percentages of circulating CD4+CD28−, CD8+CD28− and CD4+CD161+ single-positive and CD4+CD8+ double-positive T-cells in GPA. Transcriptomic profiling of sorted T-cell populations showed major differences between GPA and healthy controls reflecting antigen- (bacteria, viruses, fungi) and cytokine-driven impact on T-cell populations in GPA. Concomitant cytomegalovirus (CMV) and Epstein-Barr virus (EBV) - positivity was associated with a significant increase in the percentage of CD28− T-cells in GPA-patients compared to sole CMV- or EBV-positivity or CMV- and EBV-negativity. T-cells specific for other viruses (influenza A virus, metapneumovirus, respiratory syncytial virus) and the autoantigen proteinase 3 (PR3) were infrequently detected in GPA. Antigen-specific T-cells were not specifically enriched in any of the T-cell subsets. Altogether, on a genetic and cellular basis, here we show that alterations of the peripheral T-cell compartment are driven by inflammation and various environmental factors including concomitant CMV and EBV infection. Our study provides novel insights into mechanisms driving autoimmune disease and on potential therapeutic targets.

1. Introduction

Autoimmune disease arises in an individual from a combination of predisposing genetic and environmental factors triggering a dysregulated immune response against self, which is characterized by the break of tolerance, autoantibody production, imbalance of effector and regulatory T-cells, self-perpetuating inflammation and tissue destruction [1]. Effector molecules and cells targeting tissues

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housing the inciting autoantigen(s) maintain tissue damage and the autoimmune response. Deposition of immune complex in tissues causes further damage in many, but not all autoimmune diseases [2]. Granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis) is a prototypical autoimmune disease characterized by extravascular necrotizing granulomatous inflammation predominantly affecting the upper and/or lower respiratory tract and a necrotizing systemic autoimmune vasculitis preferentially affecting small pulmonary and renal vessels. Owing to its systemic nature, the vasculitis may however affect any organ. GPA is grouped among the anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) for its highly specific association with ANCA specific for proteinase 3 (PR3), a neutrophil- and monocyte-derived serine protease. PR3-ANCA induce near-wall neutrophil degranulation with endothelial damage *in vitro* and *in vivo.* As a result, vasculitis and vasculitic organ damage ensue representing the terminal phase of ANCA-mediated pathophysiology. AAV are characterized by lack of immune complex deposition and hence called pauci-immune vasculitides [3–5]. Notably, imbalance of effector and regulatory T-cells is a distinctive cellular feature of GPA. While the function of regulatory T-cells is impaired, circulating effector memory T-cells (T_{EM}) including Th1-type CD4+ and CD8+ T-cell populations lacking co-stimulatory CD28 expression and Th17 cells are expanded. Conversely, the percentage of circulating naïve T-cells is decreased [6–12]. Disruption of the balance between regulatory and effector T-cells and accumulation of effector T-cells in the tissues with disease propagation favour chronic inflammation and the loss of control on autoreactive T-cells [1]. Accordingly, in GPA CD28− T_{EM} are enriched in bronchoalveolar fluid, abundant in inflammatory lesions, and detectable in urine during renal activity [8,13,14].

The causes driving alterations of the peripheral T-cell compartment have remained poorly understood in GPA so far. Lower numbers of CD28− T_{EM} in the early stage of GPA as compared to late stage GPA suggest that alteration of the T-cell compartment is a dynamic and potentially metachronous process driven by multiple factors rather than a single cause [7,15]. In this study, we hypothesized that alteration of the peripheral T-cell compartment is a consequence of the impact of distinct antigen- and inflammation-driven processes. Memory T-cells comprise the antigenic experience of an individual. The information of their original inflammatory polarization conditions is imprinted on the transcriptional level [16]. Therefore, we performed phenotyping, transcriptome and functional analyses in order to test our hypothesis and disclose environmental factors and inflammatory drivers of the altered T-cell response in GPA. Our results demonstrate for the first time the impact of multiple environmental factors and inflammation on the peripheral T-cell compartment and link concomitant Cytomegalovirus (CMV) and Epstein–Barr virus (EBV) infection to the expansion of circulating CD28− T-cells in GPA. Altogether, these findings provide new insights into potential causes and mechanisms driving alterations of the T-cell compartment of an autoimmune disease at the same time.

2. Methods

2.1. Study population

All patients fulfilled the American College of Rheumatology (ACR) criteria and the Chapel Hill Consensus Conference (CHCC) definition for GPA [17,18]. Disease activity was recorded according to European League Against Rheumatism EULAR recommendations [19,20]. All patients and controls provided informed consent. The study and all experiments were performed in accordance with the declaration of Helsinki including all relevant guidelines and regulations by the ethics committee of the University of Lübeck (reference no. 07–059). Table 1 summarizes the clinical characteristics of our patient cohort. Demographic features of healthy controls (n = 20) and GPA patients (n = 20) were similar except for lower age in the healthy control group. GPA-patients had normal absolute leukocyte counts or mild leukocytosis (mean, interquartile range: 10.0 [6.6–11.8] $x10^9$/l). The majority of patients had normal lymphocyte numbers while 6 patients displayed mild to moderate lymphocytopenia (1.2 [0.6–1.9] $x10^9$/l). Yet, the CD4/CD8 T-cell ratio was similar in healthy individuals and patients with GPA (2.73 [2.06–3.72] *vs.* 2.36 [0.96–4.01], p = 0.33).

2.2. Antibodies used for flow cytometry

The following monoclonal antibodies (mAb) were used in different combinations: Alexa Fluor 488 (AF488)-conjugated anti-CD3 (clone UCHT1), Brilliant Violet 421 (BV421) or allophycocyanin (APC)-conjugated anti-CD4 (RPA-T4), peridinin chlorophyll protein complex (PerCP)-conjugated anti-CD8 (clone SK1), phycoerythrin (PE)-conjugated anti-CD45RA (clone HI100), Alexa Fluor 647 (AF647)-conjugated anti-CCR7 (clone G043H7), PE-cyanine dye 7 (PE-Cy7)-conjugated anti-CD28 (clone CD28.2), AF647-conjugated CD161 (clone HP-3G10), fluorescein isothiocyanate (FITC)-conjugated HLA-A2 (clone BB7.2), and APC- or PE-conjugated anti-NKG2D (clone BAT221) from Miltenyi Biotec (Bergisch Gladbach, Germany). All monoclonal antibodies were, unless otherwise stated, supplied by Biolegend (Fell, Germany).

2.3. Surface marker staining

Staining of cellular surface markers was performed using freshly collected whole blood (Li-heparin) as described earlier (8). Briefly, fluorochrome-conjugated mAb for cell surface antigens were added to 100 µl of whole blood and incubated for 45 min in the dark at 4 °C. Subsequently, erythrocytes were lysed by addition of FACS Lysing Solution (BD Pharmingen). After incubation for 10 min in the dark at room temperature, cells were washed twice with PBS/0.01% BSA and immediately analysed on a FACSCanto II cytometer using FACSDiva software (BD Biosciences Heidelberg, Germany).

2.4. Cell separation, RNA extraction and labelling

For transcriptional profiling of CD4+ and CD8+ single-positive and CD4+CD8+ double-positive T-cells, PBMC from GPA patients

Table 1
Patient characteristics. Localised GPA was defined as GPA restricted to the upper and/or lower respiratory tract with no constitutional symptoms. Generalised GPA was defined as GPA with renal and/or other organ-threatening disease with a creatinine <500 µmol/l (19). BVAS V3.0 = Birmingham Vasculitis Activity Index version 3.0. Remission was defined as BVAS V3.0 = 0. Disease activity in patients with either first manifestation or relapse was defined as BVAS V3.0 ≥ 1 [20]. PR3-ANCA = Anti-neutrophil cytoplasmic autoantibodies with proteinase 3 specificity. AZA = azathioprine. CYC = cyclophosphamide. LEF = leflunomide. MTX = methotrexate. RTX = rituximab. Median with interquartile range in square brackets.

Total, n	20
Age	64 [44–74]
Gender, female/male, n	4/16
Localised/generalised GPA	3/17
Disease duration (months)	35 [22–58]
Remission/active disease	16/4
BVAS remission	0
BVAS active disease	10.5 [4.5–22.5]
PR3-ANCA (±)	16/4
Prednisolone, n; dose (mg/day)	18; 5.0 [4.0–8.0]
Immunosuppressant: n	CYC: 2, RTX: 3, AZA: 5, MTX: 6, LEF: 2