Circulating CD4+CD8+ double-positive T-cells display features of innate and adaptive immune function in granulomatosis with polyangiitis

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ABSTRACT

Objective. *To examine functional features of CD4+CD8+ double-positive T-cells in patients with granulomatosis with polyangiitis (GPA) using phenotypic and transcriptomic analysis.*

Methods. *Staining of cellular surface marker was performed using freshly collected whole blood. For intracellular cytokine staining freshly collected whole blood was stimulated with phorbol myristate acetate and ionomycin. Multicolor flow cytometric analysis was performed on a FACSCanto II cytometer using FACSDiva software. Lymphocytes were gated on CD3, CD4, and CD8 staining. FACS-sorted CD4+CD8+ double-positive T-cells of GPA-patients and HC (n=3 each) were subjected to transcriptional profiling using an Affymetrix Human Genome 2.0 microarray. Differently expressed genes were analysed using biological databases.*

Results. *Frequency of CD4+CD8+ double-positive T-cells was increased within the total CD3+ T-cell population in GPA, but no difference was detected between patients with active disease and remission. Percentages of interferon γ (Th1-type), interleukin 17 and interleukin 22 (Th17-type) producing CD4+CD8+ double-positive T-cells exceeded the percentage of interleukin 4 (Th2-type) producing cells. There were no significant differences in the percentages of the respective cytokine-positive CD4+CD8+ double-positive T-cells between GPA and HC. Up-regulated genes of CD4+CD8+ double-positive T-cells in GPA were enriched within Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to nuclear factor kappa-light-chain-enhancer of activated B cells signalling, toll-like receptor signalling, nucleotide-binding oligomerisation domain-like receptor signalling as well as major histocompatibility complex class-II antigen presentation.*

Conclusion. *Employing a combined phenotypic and transcriptomic approach we disclosed a Th1/Th17 phenotype as well as innate and adaptive functions of CD4+CD8+ double-positive T-cells in GPA.*

Introduction

Following the developmental stage of $CD4^-CD8^-$ double-negative cells, T-cells pass through the $CD4^+CD8^+$ double-positive (DP) cell stage during thymopoiesis (1). Finally, after positive and negative intrathymic selection processes resulting in the expression of T-cell receptors (TCR) with low self-affinity and transcriptional repression of the opposing co-receptor CD8 and CD4, respectively, T-cells leave the thymus as naïve $CD4^+$ and $CD8^+$ single-positive cells (1, 2). Therefore, in healthy individuals, only few DP T-cells are detected in peripheral blood (3). However, an increased frequency of circulating DP T-cells has been reported in association with different infections and in chronic inflammatory and autoimmune diseases (3-5). Data from experimental studies suggest that circulating DP T-cells are not recent thymic emigrants, but originate from $CD4^+$ and $CD8^+$ single-positive memory cells re-expressing the other co-receptor, *i.e.* CD8 or CD4, under conditions of chronic antigen- and/or cytokine-stimulation (5-7). Recently, we showed an expansion of circulating DP T-cells in patients with granulomatosis with polyangiitis (GPA), an anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV). DP T-cells displayed a memory cell phenotype in GPA (8). Transcriptome analysis showed 352 differentially expressed genes (DEG) in DP T-cells in GPA compared with those of healthy controls. These DEG contained genes encoding cell-surface receptors, tran-

scription factors, intracellular signalling proteins, calcium-binding proteins, cytokines/chemokines, major histocompatibility complex (MHC) proteins, and adhesion molecules. Gene ontology analysis of DEG showed nuclear factor-κB (NF-κB), toll-like receptors (TLR), cytokine/cytokine receptor interaction and chemokine cell signalling pathways as well as pathogen-related pathways to be enriched in GPA (8).

In the present study, we further analysed functional features and the transcriptomic profile of DP T-cells from the peripheral blood of that group of patients with GPA presented in our previous study (8). Here, we now demonstrate that DP T-cells predominantly produce Th1-type and Th17 cytokines. Moreover, DP T-cells display transcriptional expression of genes related to innate and adaptive immune functions including nucleotide-binding oligomerisation domain, leucine rich repeat and pyrin domain containing (NLRP)3, NLRP12, toll-like receptor (TLR) expression and major histocompatibility complex (MHC) class II presentation. Signalling pathways were closely connected as demonstrated and visualised using biological pathway and interaction databases Reactome and Search Tool for the Retrieval of Interacting Genes and Proteins (STRING).

Much effort has been made to understand the pathophysiology of AAV, such as GPA (9). However, there is still a need for new disease-related biomarker and targets for improving therapies for AAV. Therefore, the present work could contribute to the development of novel T-cell-directed treatment strategies for GPA.

Materials and methods

Study population

We have presented our study population in a recent report (8). All patients fulfilled the American College of Rheumatology (ACR) criteria and the Chapel Hill Consensus Conference (CHCC) definition for GPA (10, 11). Disease activity was recorded using the Birmingham Vasculitis Activity Score (BVAS) v. 3.0 (12). The study was approved by the local ethics committee (no. 07-059). The patients' characteristics are summarised in Table I.

Table I. Patient characteristics.

Total number of patients	20
Age (years; median, range)	64 [33 – 83]
Sex (f/m)	4/16
Disease duration (months; median, range)	35 [1 – 178]
Localised GPA	3
Generalised GPA	17
PR3-ANCA positive patients	16
Patients with active disease	4
BVAS in active disease (median, range)	10.5 [4 – 29]
GPA in remission	16
BVAS in remission	0
Prednisolone	18
Prednisolone dosage (mg/day; median, range)	5 [2.5 – 80]
CYC//RTX/AZA/MTX/LEF	2/3/5/6/2
No treatment	2

If not specified otherwise, the number of patients concerned is noted.
AZA: azathioprine; BVAS: Birmingham Vasculitis Activity Index version V3.0; CYC: cyclophosphamide; GPA; granulomatosis with polyangiitis; MTX: methotrexate; LEF: leflunomide; PR3-ANCA: anti-neutrophil cytoplasmic autoantibodies with proteinase 3 specificity; RTX: rituximab.

Flow cytometric analysis

Cells from whole blood were sorted using a fluorescence-activated cell sorter (FACSCanto II) and analysed using FACSDiva software (BD Biosciences Heidelberg, Germany). Staining of cellular surface marker was performed using freshly collected whole blood. For intracellular staining, cells were stimulated with phorbol myristate acetate (PMA, Sigma, Munich, Germany) (10ng/ml) and ionomycin (Sigma) for 4 hours. Cytokine secretion was inhibited with Brefeldin (Sigma). After staining of surface antigens, cells were fixed and permeabilised, and intracytoplasmatic cytokine staining was performed as described previously (8). The following antibodies were used for staining in different previously determined optimal concentrations and 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), PE-conjugated anti-CD24, FITC-conjugated tdt from BD Biosciences (Heidelberg, Germany), APC-Cy7-conjugated anti-interferon (IFN)-γ, PE-Cy7-conjugated anti-interleukin (IL)-4, PE-conjugated anti-IL-17a from eBioscience (Frankfurt, Germany), APC-conjugated anti-IL-22 from R&D Systems (Wiesbaden, Germany). All monoclonal antibodies were, unless otherwise stated, supplied by Biolegend (Fell, Germany).

Transcriptome and biological database analysis

FACS-sorted T-cells from GPA-patients and HC (n=3 each) were subjected to transcriptional profiling using an Affymetrix Human Genome 2.0 microarray, as previously described and performed. Purity of sorted $CD4^+$ single-positive and $CD8^+$ single-positive T-cell was ≥98% and DP T-cell ≥97% (8). Enrichment and pathway analysis of DEG were further categorised by GO functional enrichment analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) PATHWAY database as described elsewhere (8). In the present study, biological processes and pathways of DEG were analysed with Reactome Knowledgebase (13). Functional protein-protein interaction networks were analysed, clustered and visualised by the STRING (14).

Statistical analysis

Statistics were performed using Prism 4.0 (GraphPad Software, San Diego, CA, USA). Data are presented as means with standard error of mean (SEM) or as indicated. Gaussian distribution was tested using the Kolmogorov-Smirnov test. The unpaired *t*-test was used to compare data from GPA-patients with

those from healthy controls and between patients with active disease and in remission. Two-tailed *p*-values of less than 0.05 were regarded as statistically significant.

Results

Frequency and cytokine profile of circulating CD4+CD8+ double-positive T-cells

The frequency of DP T cells within the total CD3+ T-cell population in peripheral blood was increased in GPA-patients compared with healthy individuals (Fig. 1A, left two bars) as reported earlier (8). In the present study, we found no statistically significant difference in the frequency of circulating DP T-cells between GPA-patients with active disease and in remission (Fig. 1A, right two bars).

Under experimental conditions, circulating DP T-cells were shown to originate from CD4+ and CD8+ single-positive memory cells re-expressing the other co-receptor, *i.e*. CD8 and CD4, respectively (5). Therefore, we hypothesised that circulating DP T-cells lack expression of the sialoglycoprotein CD24 and terminal deoxynucleotidyl transferase (tdt) characteristic of immature thymocytes (1, 15-17). In our present study, DP T-cells from peripheral blood lacked CD24 and tdt expression in both, healthy individuals and patients with GPA (3.0±0.3% *vs*. 2.8±0.6 and 1.2±0.3% *vs*. 0.7±0.3% in healthy controls *vs*. GPA, respectively, data not shown). Next, we analysed whether double-positive T-cell were cytokine-producing cells suggestive of memory cell differentiation and function. To investigate cytokine production of DP T-cells, we analysed interferon (IFN)-γ, interleukin (IL)-4, IL-17 and IL-22 production of gated DP T-cells within the total CD3 T-cell population following PMA/ionomycin stimulation. We found, that the frequency of interferon (IFN)-γ, interleukin (IL)-17 and IL-22 producing DP T-cells exceeded the percentage of IL-4 producing cells in healthy individuals and GPA. There were no significant differences in the percentages of IFN-γ, IL-4, IL-17 and IL-22 producing DP T-cells between healthy controls and GPA-patients (Fig. 1B).

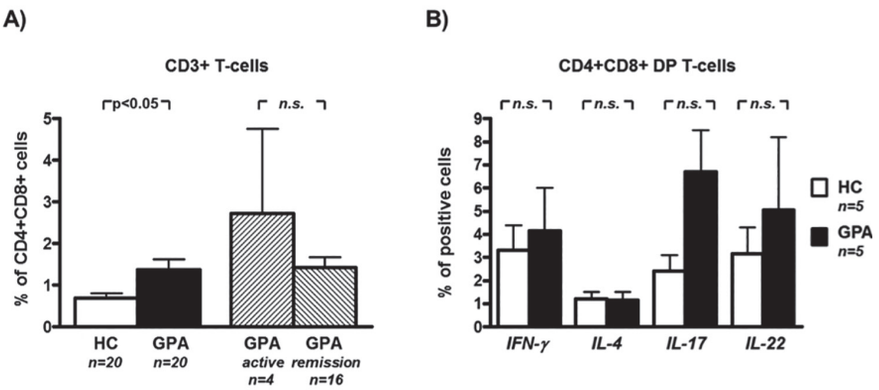

Fig. 1. Frequency of CD4+CD8+ double-positive T-cells within the CD3+ T-cell population and cytokine producing cells within the double-positive T-cell population. **A)** Percentage of CD4+CD8+ double-positive cells within the CD3+ T-cell population in healthy controls and GPA (left two bars) and in GPA-patients with active disease and in remission (right two bars). **B)** Percentages of IFN-γ, IL-4, IL-17 and IL-22 producing cells within the CD4+CD8+ double-positive cell population in healthy controls and GPA. Bar charts with mean and error bars representing standard error of the mean (SEM). *P*-values were calculated using the unpaired *t*-test. n.s. = no significant difference.

Transcriptional up-regulation of genes linked to innate and adaptive immune responses in CD4+CD8+ double-positive T-cells

In a previous study we compared the transcriptome profile of sorted CD4+ and CD8+ single-positive and DP T-cell populations from GPA-patients to those from healthy controls (8). We identified 352 DEG in DP T-cell (48 downregulated and 304 upregulated genes) in GPA (8). In the present study, we investigated underlying biological processes and functional relationships by submitting the list of up-regulated genes to the Reactome Knowledgebase, an over-representation analysis tool for interpretation and visualisation of expression data sets (13). We found an unexpected significant over-representation of up-regulated genes within innate immune pathways (129 found entities of 2548 total entities, p=0.000176, false discovery rate = 0.0173). Indicated over-represented entities from the (innate and adaptive) immune system were further submitted to STRING database to identify protein–protein interaction networks, which revealed 140 items within the presented network. To gain further information of relevant modules we used the Markov Clustering (MCL) algorithm within the STRING tool that represents a robust clustering method for extraction of complexes from interaction networks (18) (Fig. 2).

Twenty-five clusters were calculated and allocated to distinct colours (Supplementary Table). In addition, functional enrichment was observed within relevant KEGG pathways, namely NFκB signalling, TLR signalling, NLR signalling as well as antigen processing and presentation (Fig. 3).

We detected a great overlap of genes belonging to these particular KEGG pathways and distinct clusters from the MCL calculation (NFκB: cluster 1; TLR signalling: cluster 3; antigen presentation: cluster 5, NLR signalling: cluster 7).

Discussion

In this study, we present data from our further work-up of functional aspects and the transcriptomic profile of DP T-cells in GPA. Previously, we have shown an expansion of DP T-cells in peripheral blood in GPA. In contrast, there was no increase in the frequency of CD4-CD8- double-negative T-cells (8). In the present study, we found no difference in the frequency of circulating DP T-cells between GPA-patients with active disease and in remission. DP T-cells lacked expression of marker suggestive of recent thymic emigration. These cells produced predominantly Th1-type and Th17 cytokines. Notably, analysis of the transcriptome disclosed the expression of genes related to adaptive as well as innate immune functions and MHC class II presentation.

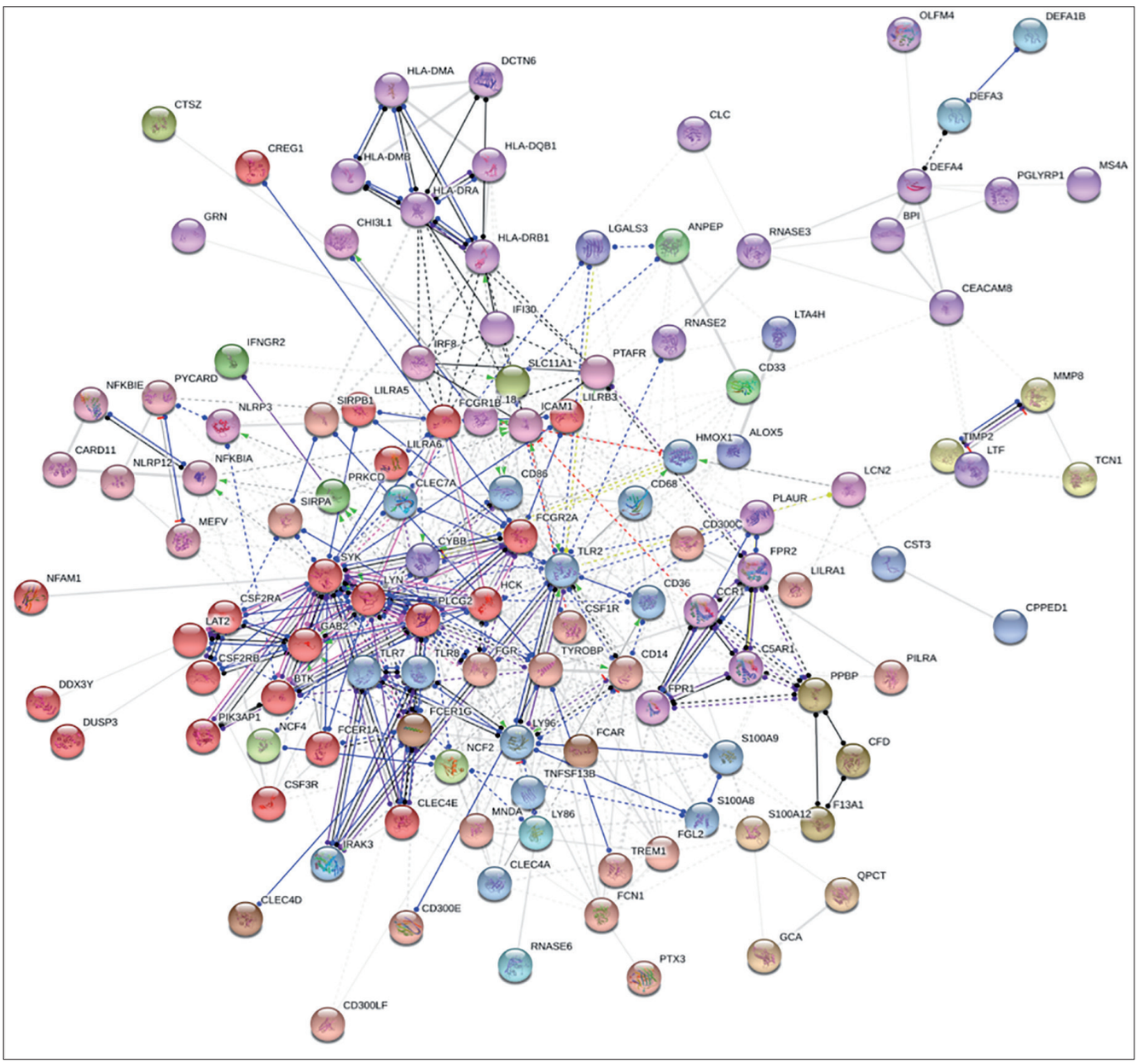

Fig. 2. Protein-Protein interaction network of up-regulated genes in of CD4+CD8+ double-positive T-cells. Over-represented entities from the (innate and adaptive) immune system of the Reactome analysis (n=140) were submitted to STRING database to identify protein–protein interaction networks (PPI enrichment *p*-value: <1.0e-16). Distinct colors represent clusters calculated by Markov Clustering (MCL) algorithm within the STRING tool (see Supplementary Table).

Expansion of circulating DP T-cells has been reported in various infectious, non-infectious chronic inflammatory and autoimmune diseases (5). As reported earlier, an increased frequency of DP T-cells is also found in peripheral blood GPA-patients (8). Here, we found no significant difference in the frequency DP T-cells between active disease and remission. Still, the frequency of DP T-cells was numerically higher in active GPA. The lower number of patients with active disease compared to patients in remission could have contributed to the failure of finding a significant difference in the frequency of DP T-cells in this group of GPA-patients.

In our previous study, we showed that circulating DP T-cells predominantly display a memory cell phenotype similar to the phenotype of circulating DP T-cells reported in different chronic viral and autoimmune diseases (3-5, 8). Here we show that DP T-cells lack CD24 and tdt expression. Both markers are expressed by thymocytes during thymopoiesis. CD24 plays a role in the negative selection and tdt in TCR repertoire generation of thymocytes. Both markers are down-regulated before T-cells exit the thymus (15-17, 19). Lack of CD24 and tdt expression by DP T-cells suggests that circulating DP T-cells are not immature recent thymic emigrants. Our finding is consistent with earlier experimental studies suggesting that circulating DP T-cells originate from CD4+ and CD8+ single-positive memory cells re-expressing the other co-receptor, *i.e.* CD8 or CD4, under conditions of chronic antigen- and/

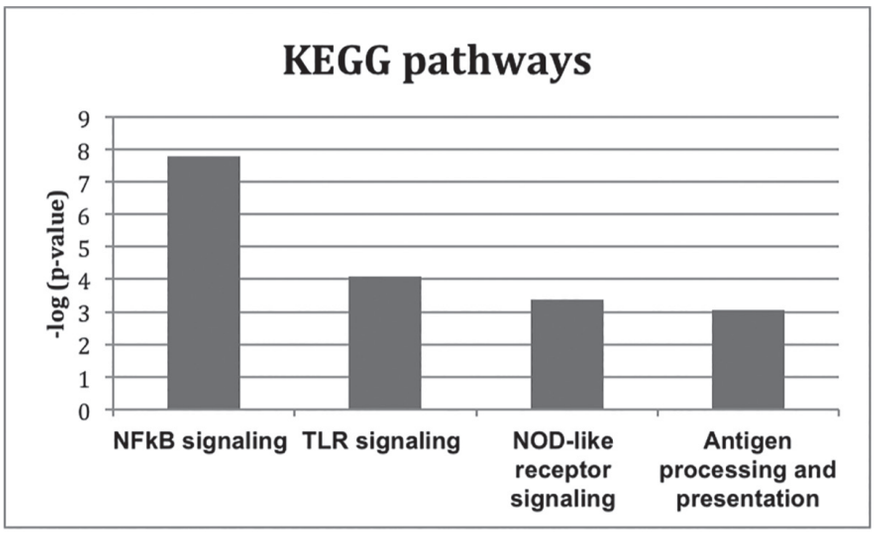

Fig. 3. Functional enrichment analysis of $CD4^+CD8^+$ double-positive T-cell gene signature in GPA. Functional enrichment in relevant KEGG pathways within the STRING protein-protein interaction network of $CD4^+CD8^+$ double-positive T-cells.

or cytokine-stimulation (5-7). In line with a memory cell origin of DP T-cells and antigen- and polarising cytokine-driven epigenetic imprinting (5, 20, 21), we found that DP T-cells predominantly produced IFN-γ, IL-17 and IL-22, *i.e*. Th1-type and Th17 cytokines, upon stimulation.

As already described by transcriptomic profiling of single- and double-positive T-cells, we detected major differences between GPA and healthy controls, reflecting environment and inflammation-driven T-cell activation (8). Additional analysis of DEG of DP T-cells using several biological database analysis tools identified the involvement of innate (NLR and TLR signalling) and unexpected adaptive cellular processes (MHC class II antigen presentation). It has been shown before that activated human T-cells are indeed capable of expressing MHC class II molecules of all isotypes (HLA-DR, HLA-DQ, and HLA-DP) as well as to process and present antigen (22). This might represent a mechanism of T-cell homeostasis, as MHC class II ligation on these T-cells could induce anergy or apoptosis. Whether DP T-cells are functioning as antigen presenting cells (so called T-APC) in GPA has to be further investigated. Moreover, we identified increased expression of genes related to the inflammasome pathway (NLRP3, IL-18) in GPA. It has been demonstrated, that the NLRP3 inflammasome together with intracellular activation of complement plays an important role in Th1 induction in single-positive $CD4^+$ T-cells (23). Furthermore, the interplay between the NLRP3 inflammasome and T-cell populations were linked to inflammatory processes in human adipose tissue inflammation (24).

In conclusion, DP T-cells display phenotypic and functional features of memory T-cells. In GPA, DEG are upregulated in DP T-cells. Transcriptomic profiling discloses upregulation of pathways related to NFkB signalling, TLR signalling, NOD-like receptor signalling, and antigen processing and presentation, *i.e*. both innate and adaptive immune functions. Dual innate and adaptive function of DP T-cells may curtail their need for restimulation in lymphoid organs and thereby save time in the presence of chronic antigenic challenge. In GPA, expansion of a circulating DP T-cell population with a self-sustaining hybrid T-APC-like function may, thus, represent an escape mechanism of the immune system in order to deal with otherwise overwhelming chronic stimulation in autoimmune disease.

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