LONG-TERM EFFECT OF HIGH CYCLOPHOSPHAMIDE DOSES ON THE REPERTOIRE OF T-CELL RECEPTORS OF PERIPHERAL BLOOD T-LYMPHOCYTES IN PATIENTS WITH AUTOIMMUNE VASCULITIS

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Although mechanisms underlying development of autoimmune vasculitis and polyangiitis remain understudied, these pathologies are already known to be largely mediated by T-lymphocytes. Cyclophosphamide (CF) is widely used to treat autoimmune diseases. Lymphoid cells in general (T, B, and NK cells) and naive T-lymphocytes in particular are highly sensitive to CF. In this work we analyzed the repertoires of T-cell receptors (TCRs) in the peripheral blood of young (aged 24 to 35 years, n = 4) and elderly (aged 52 to 68 years, n = 5) patients with ANCA-associated vasculitis (Wegener granulomatosis and Churg–Strauss syndrome) treated with high doses of CF > 3 years before the study. The control group included 7 young and 14 elderly healthy individuals. We revealed no TCR variants previously reported as typically found in patients with ANCA-associated vasculitis. Relative frequency of “public" (often found in a population, largely formed during an embryonic period) TCR variants in the repertories of young patients was significantly lower than in the repertories of healthy donors of the same age, and was similar to the elderly healthy donors. We hypothesize that CF-treatment eliminates substantial proportion of naïve T-cells in the young donors, that contains "public" TCR variants of fetal origin. Long-term consequences of such changes in the structure of T-cell immunity require further investigations.

Keywords: autoimmune disease, autoimmune vasculitis, cyclophosphamide, T-cell receptor, naive T-lymphocyte, high-throughput sequencing

Funding: this work was supported by the Russian Science Foundation (Grant No. 16-15-00149). Equipment used in the study was provided by the shared facility of the Institute of Bioorganic Chemistry (the facility is supported by the Ministry of Education and Science of the Russian Federation, project ID RFMEFI62117X0018).

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Received: 04.10.2017 **Accepted:** 25.10.2017

ИССЛЕДОВАНИЕ ДОЛГОСРОЧНОГО ЭФФЕКТА ВЫСОКИХ ДОЗ ЦИКЛОФОСФАМИДА НА РЕПЕРТУАР Т-КЛЕТОЧНЫХ РЕЦЕПТОРОВ Т-ЛИМФОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ У ПАЦИЕНТОВ С АУТОИММУННЫМИ ВАСКУЛИТАМИ

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Механизмы развития аутоиммунных васкулитов и полиангиитов мало изучены, однако известно, что патогенез этих заболеваний в значительной степени опосредуется Т-лимфоцитами. Циклофосфамид (ЦФ) широко используется для лечения аутоиммунных заболеваний. Клетки лимфоидного ряда (Т, В и NK-клетки), и в особенности наивные Т-лимфоциты, обладают высокой чувствительностью к ЦФ. Мы проанализировали репертуары Т-клеточных рецепторов (T-cell receptors, TCRs) периферической крови молодых (24–35 лет, n = 4) и пожилых (52–68 лет, n = 5) пациентов с синдромами Вегенера и Чарга–Стросса, получавших не ранее чем за 3 года до начала исследования ЦФ в высоких дозах. В контрольную группу включили здоровых доноров: 7 молодых и 14 пожилых людей. Мы не выявили описанных ранее вариантов TCRs, характерных для ANCA-ассоциированных васкулитов. Представленность «публичных» (часто встречающихся в популяции, в значительной степени формирующихся в эмбриональном периоде) вариантов TCRs в репертуарах молодых пациентов оказалась существенно ниже, чем в репертуарах здоровых доноров того же возраста, и была близка к таковой пожилых здоровых доноров. Мы предполагаем, что терапия высокими дозами ЦФ элиминирует значительную часть наивных Т-лимфоцитов молодых доноров, содержащих публичные варианты TCR эмбрионального происхождения. Отдаленные последствия таких изменений в структуре Т-клеточного иммунитета требуют дальнейшего изучения.

Ключевые слова: аутоиммунные заболевания, аутоимунный васкулит, циклофосфамид, Т-клеточный рецептор, наивный Т-лимфоцит, высокопроизводительное секвенирование

Финансирование: работа выполнена при поддержке Российского научного фонда, грант № 16-15-00149. В исследовании было использовано оборудование Центра коллективного пользования ИБХ РАН, поддержанного Министерством образования и науки России, идентификатор соглашения — RFMEFI62117X0018.

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Статья получена: 04.10.2017 **Статья принята к печати:** 25.10.2017

Immunosupressants have become a common treatment option for autoimmune diseases. One of such drugs, the cytotoxic cyclophosphamide (CPH), whose immunosuppressive properties are still understudied, is used for treating severe pathologies, including autoimmune vasculitis. CPH is metabolized in the liver into 4-hydroxycyclophosphamide that readily diffuses into the cells and then converts into either phosphoramide mustard, an active cytotoxic compound, or inert carboxyphosphamide (given that the cell is rich in aldehyde dehydrogenase). Lymphoid cells, including T- and B-lymphocytes and natural killer cells, are low in aldehyde dehydrogenase and die when exposed to high doses of cyclophosphamide. But primitive hematopoietic cells are rich in this enzyme and therefore resistant to CPH. Thus, CPH has a pronounced immunosuppressant effect, but is not myeloablative: stem cells survive exposure to CPH, retaining their hematopoietic activity, which renders bone marrow transplantation irrelevant [1]. High doses of CPH have been clinically confirmed to induce remission in patients with different types of autoimmune disorders (acute aplastic anemia, myasthenia gravis, systemic scleroderma, etc.), but 5-year relapse-free rates following the treatment with CPH are as low as 10 % [2].

There are reports about the specific effect of CPH on naive T-cells. For example, Gladstone et al. [3] have demonstrated a significant reduction in naive $CD45RA^+CD4^+$ T-lymphocytes in comparison with $CD45RO^+CD4^+$ memory cells in patients with multiple sclerosis who received high doses of CPH. Almost all the participants retained their immune status, including the resistance to the infections they had had in childhood, which is mediated by memory cells. Gladstone's findings indicate that memory cells are less sensitive to CPH than naive T-lymphocytes.

The evolution of high-throughput sequencing has enabled deep profiling of T-cell receptors (TCR) repertoire. Combined with an immunofluorescence staining, this technology has yielded a few important discoveries about age-driven changes in the adaptive immunity. In our previous work we have analyzed a wide range of samples, including umbilical cord blood and peripheral blood of centenarians, to reveal a correlation between the reduction in naive T-lymphocytes and the decreasing diversity of the TCR repertoire. We have also described some changes in the structure of the TCR repertoire occurring throughout the life [4].

Mechanisms underlying the development of vasculitis remain understudied. The primary diagnostic marker for this disease is anti-neutrophil cytoplasmic antibodies (ANCAs). Patients with Wegener granulomatosis (granulomatosis with polyangiitis, GPA) have antibodies to proteinase-3 (PR-3), while patients with Churg–Strauss syndrome (eosinophilic granulomatosis with polyangiitis, EGPA) and microscopic polyangiitis have antibodies to myeloperoxidase. ANCAs are thought to be implicated in the pathogenesis of vasculitis through the interaction with the antigen and stimulation of neutrophil degranulation, which causes endothelial and therefore vascular damage [5]. T cells also actively contribute to the development of the disease. The afflicted patients have increased counts of activated $CD4^+$ and $CD8^+$ T-lymphocytes circulating in the peripheral blood and elevated levels of proinflammatory factors implicated in their activation. Activated T-cells are also known to participate in granuloma formation [6, 7]. It has been shown that glomerular crescent formation is suppressed in animals with depleted T-lymphocytes [8].

A question remains about the effect of CPH on different functional subsets of blood cells and its long-term impact on the adaptive immunity. In the recent years a number of works have been published concerning the role of CPH in TCR reconstitution after allogeneic blood or marrow transplantation. It has been shown that high doses of CPH, administered on days 3 and 4 after the intervention, significantly reduce the risk of both acute and chronic "graft-versus-host" disease and viral infections, including Epstein-Bar-related lymphoproliferative posttransplantation conditions [9]. Kanakry et al. demonstrate that the first to recover after CPH treatment are effector memory cells whose diversity is, however, impoverished in comparison with that of healthy donors' cells [10].

In this work we employed high-throughput sequencing to study repertoires of peripheral blood TCR obtained from 9 patients with vasculitis previously treated with high doses of cyclophosphamide. Specifically, we compared TCR beta-chains repertoires in these patients and healthy controls and attempted a search for disease-associated variants of TCR and expression patterns of well-represented V- and J-beta TCR segments. We also evaluated the long-term effect of CPH on the repertoire of peripheral blood TCR.

METHODS

Selecting the participants

Two groups were formed: a group of patients diagnosed with vasculitis who had undergone treatment with high doses of cyclophosphomide > 3 years before the study and a group of healthy volunteers (controls). Each group was divided into subgroups of young (24–35 years) and elderly (52–68 years) individuals. The subgroup of young patients consisted of 3 males and 1 female, the subgroup of elderly patients included 4 males and 3 females; the control group consisted of 6 males and 8 females.

The study was approved by the Ethics Committee of Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology, Moscow (Protocol No. 2013-5/4). Informed consent was obtained from all participants.

Isolation of mononuclear cells and immunofluorescence staining

Eight milliliters of blood were collected from each participant for blood count and immunofluorescence staining performed as described in [11]. A hundred microliters of peripheral blood were incubated with the following monoclonal antibodies: FITC anti-human CD45RA (eBioscience, Thermo Fisher Scientific, USA), CD27-PC5 (eBioscience), CD4-PE (Beckman Coulter, USA), and CD8-eFluor 405 (eBioscience), and then lysed using Optilyse-C (Beckman Coulter). The samples were analyzed in the flow cytometer Cytomics CF500 (Beckman Coulter). Peripheral blood mononuclear cells were isolated from 6 ml of blood by Ficoll-Paque density gradient centrifugation (PanEco, Russia).

Isolation of total RNA, preparation of TCR cDNA libraries and sequencing

Total RNA was isolated using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA yield was measured in the QuBit 3 fluorometer (Invitrogen) by intercalator fluorescence intensity. Reaction quality was evaluated by gel electrophoresis.

TCR cDNA libraries were obtained as described in [11]. TCR beta chain cDNA was synthesized using the Mint kit

(Evrogen, Russia) according to the manufacturer's protocol and 1.5 μg of RNA per reaction. Depending on the amount of the isolated RNA, 4 to 6 synthesis reactions were run per sample. The primers used for cDNA synthesis, the 5′-template switch adaptor carrying 12 random nucleotides and the amplification protocol are described in [11]. Owing to the use of the 5′-adaptor, each synthesized cDNA received a unique barcode. The schematic of the experiment is shown in Fig. 1.

The obtained cDNA was amplified, purified using the Cleanup Standard kit (Evrogen) and concentrated. Concentrations of the obtained libraries were measured in QuBit 3 (Invitrogen). cDNA molecules were ligated to Illumina TruSeq adaptors (Illumina, USA). Libraries were sequenced using Illumina HiSeq 2000 (Illumina) to generate 2 x 150 nt paired-end reads.

Bioinformatic analysis of sequencing data

Demultiplexing of sequencing data and assembly of reads grouped by their molecular identifier (barcode) were done using the MiGEC pipeline [12] as described in [11]. CDR3 extraction and assembly of T-cell clonotypes were aided by the MiXCR software [13]. Qualitative analysis of clonotypes (V- and J-usage, repertoire overlap) and diversity estimates were conducted using VDJtools [14].

Data were statistically processed in the GraphPad Prism5 (Graph Pad Software, USA). Public clonotypes were analyzed using R algorithms.

RESULTS

Flow cytometry of peripheral blood T-cell subsets

Flow cytometry was used to quantify peripheral blood naive $CD3^+$ cells (phenotype $CD3^+CD45RA^+CD27^+$) and naive $CD4^+$/$CD8^+$ lymphocytes in the samples of patients with vasculitis treated with high doses of CPH. The results were compared to previously obtained data on healthy donors of different ages [4, 11]. No significant difference was observed in the proportion of naive T-lymphocytes between diseased and healthy individuals of the same age (Fig. 2, A).

Analysis of TCR diversity by high-throughput sequencing

Molecular barcoding of cDNA libraries allowed us to trace and correct substitution errors occurred during amplification and sequencing. To prepare cDNA libraries necessary for the analysis of TCR repertoires, we used cap switching technology and employed specific synthesis primers [4] (Fig. 1). Cap switching ensures uniform amplification of fragments that correspond to different T-cell receptors, thus preventing misrepresentation of variable gene segments. Each cDNA library was prepared using the entire amount of total RNA isolated from 3.5 billion mononuclear cells. The obtained total cDNA was used for amplification in full. Sequencing yielded over 30 million reads, the minimum number of reads was 1.3×10^6, the maximum number was 6.5×10^6. Molecular identifiers introduced during the cDNA synthesis stage were used as a filter during the analysis (Fig. 1): only those uniquely labeled cDNA molecules that were covered by at least 3 reads were factored into. The analysis yielded 27,000 to 400,000 read groups each carrying a unique barcode and corresponding to a unique cDNA molecule.

Sequencing data was normalized by random selection of 25,000 cDNA events per sample, i. e. to the size of the smallest dataset obtained from a patient's sample. It means that further analysis was performed on 25,000 unique TCR beta chain cDNA molecules representing each sample. Previously analyzed TCR repertoires of healthy individuals [11] were used as a reference. The reference dataset was also downsized to 25,000 random cDNA events per sample. Our previous experiments demonstrated that one cDNA event is on average equivalent to one T-cell [15]. Thus, the diversity of a T-cell repertoire was inferred from the analysis of 25,000 random peripheral blood T-lymphocytes.

The following metrics were used to estimate the diversity of the TCR beta chain repertoire: the lower bound for species richness (the Chao1 estimator), the Shannon-Wiener index of even distribution and the observed diversity of CDR3 (per 25,000 T-lymphocytes).

No significant differences were found in TCR repertoire profiles between patients with vasculitis and healthy donors (Fig. 2, B–D). But although the proportions of naive T-cells in the peripheral blood of diseased and healthy individuals were almost the same, Chao1 values were lower for patients with vasculitis (Fig. 2, B, E), indicating the depletion of the TCR diversity.

Analysis of beta-chain variable segments

Normally, the role of clonal T-cell populations in pathology is estimated by spectratyping (analysis of CDR3 fragment lengths in general and TCR beta chain V-segments in particular).

A few researchers used TCR spectratyping to demonstrate involvement of clonal and oligoclonal T-cells carrying a particular V-segment in GPA [18] and EGPA [17]. Later, TCR V-segments were found to be overrepresented in the peripheral blood of patients with EGPA in another flow cytometry-based study [23].

These findings inspired the search for the described TCR V-segments in patients with GPA and EGPA, prompting us to analyze the frequencies of different beta-chain segments in the diseased and healthy individuals. We revealed no specific expression patterns of V- or J-segments that could divide TCR repertoires observed in afflicted patients or healthy controls into distinct clusters.

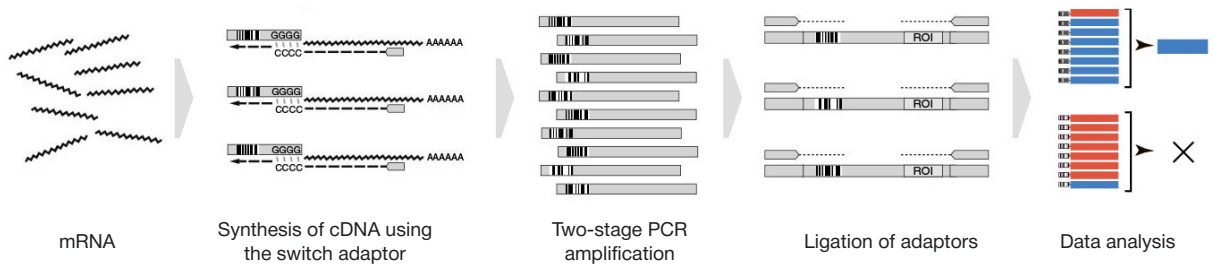

Fig. 1. Preparation of TCR cDNA libraries. The switch adaptor carries a unique sequence (shown as a barcode in the picture) which allows accurate error correction at the stage of data analysis. The amplified fragment carries TCR beta chain CDR3 responsible for receptor diversity

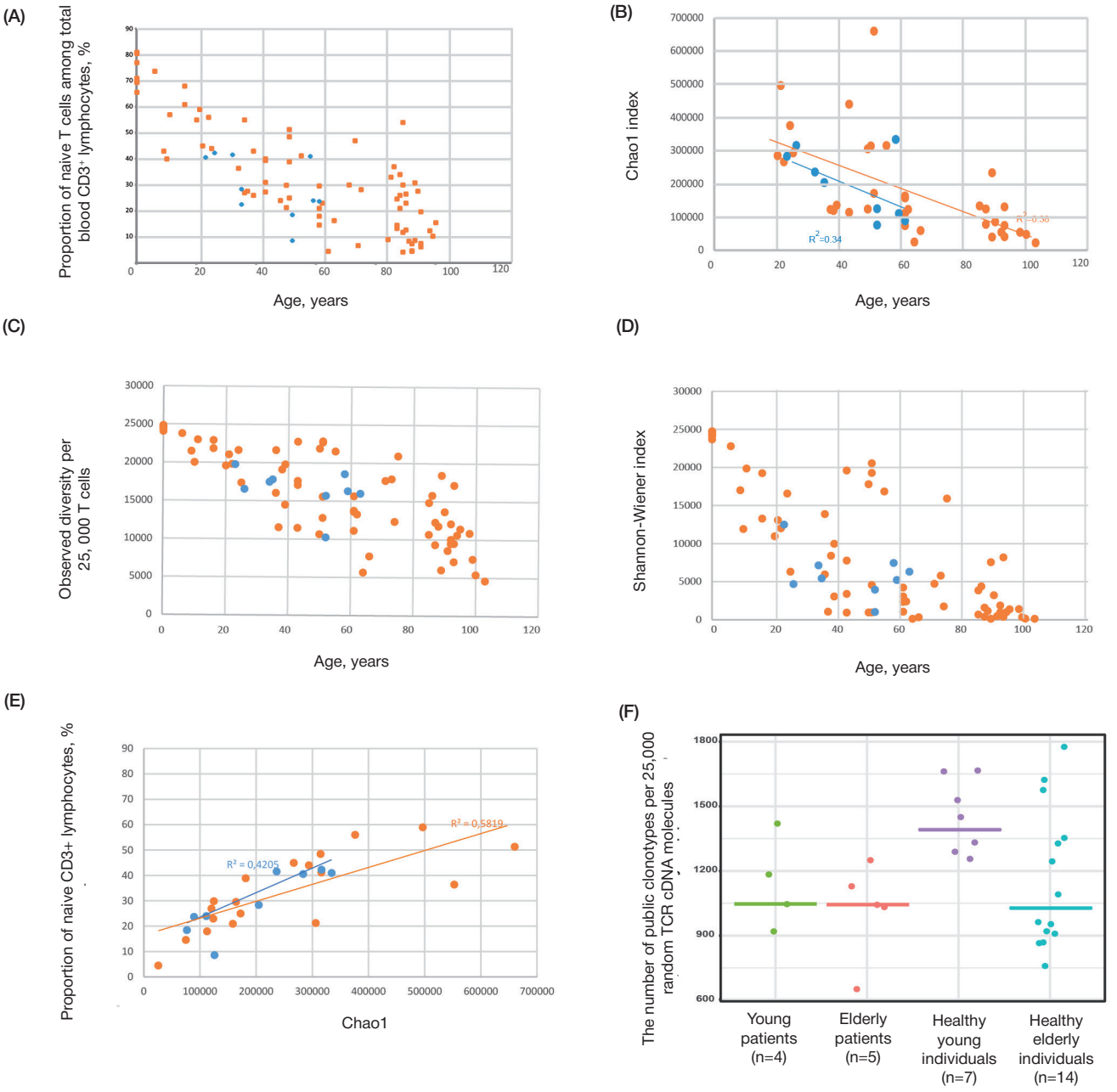

Fig. 2. The proportion of naive T cells among the peripheral blood CD3+ lymphocytes and the diversity of TCR beta chain repertoires in patients with autoimmune vasculitides who previously received high doses of cyclophosphamide (blue dots) and healthy individuals (orange dots) **(A)** The proportion of naive T cells among the total CD3+ lymphocytes plotted against participants' age. **(B)** The diversity of TCR estimated with Chao1. The major contribution is made by low-frequency clones, i.e. TCRs of naive lymphocytes. **(C)** The diversity of CDR3 sequence variants per random 25, 000 TCR beta chain cDNA molecules **(D)** The diversity of TCR beta chains estimated with the Shannon-Wiener index used to measure the evenness of clonotype distribution in the TCR repertoire. **(E)** The proportion of naive T cells among total CD3+ lymphocytes, accounting for Chao1. **(F)** The number of public clonotypes in clone sets depending on donor's age. Fig. B-E are based on the analysis of 25,000 randomly selected TCR beta chain cDNA molecules

Search for GPA- and EGPA-associated CDR3 sequences

Next, we analyzed the sequencing data of our patients for the presence of the annotated CDR3 sequences associated with both GPA and EGPA [17, 18, 23]. We searched in unnormalized clone sets. We did not find any CDR3 motifs previously announced to be GPA — or EGPA —associated.

Estimation of the proportion of public clonotypes in TCR repertoires

High-throughput sequencing of immune repertoires is very instrumental in obtaining individual lists of TCR sequences the organism employs to develop adaptive immune response. Among the millions of TCR variants sequenced by the authors of this work and other researchers, the so-called public (or non-unique) TCR clonotypes have been identified, occurring repeatedly in the immune repertoires of unrelated participants [11, 19, 20]. Identical TCR sequences found in unrelated individuals are thought to be generated through stochastic recombination of TCR gene segments or in an attempt to produce optimal TCR clones for effective immune response to a widespread pathogen or as a result of TCR co-evolving with a persisting infection transmitted through generations. Relatives share more TCR clonotypes than unrelated donors. On the whole, the genetic environment of a

donor cohort can affect the outcome of the analysis of public TCR repertoires.

In our previous work we showed that the proportion of public TCR clonotypes decreases with age [4]. In other words, it is a marker of TCR repertoire ageing. Previously we drew a list of human TCR beta-chain clonotypes present in the T-lymphocyte repertoires of different donors. Public clonotypes were extracted from the sequences of highly diverse TCR repertoires of umbilical cord blood where they are abundant. We selected clonotypes with the identical CDR3 amino acid sequence found in at least 4 of 8 samples of umbilical cord blood obtained from healthy donors. This list of clonotypes was further supplemented with short high-frequency CDR3 variants that are generated during VDJ recombination, primarily during fetal development [16], and sequences without random N-nucleotide insertions at the V–D or D–J junctions resulting from a simple VDJ rearrangement in embryos in the absence of TdT-transferase expression.

Public clonotypes of TCR beta chains emerged in the embryonic stage often coincide with the list of super public TCR variants that can be observed in healthy adults (8 to 85 years, n = 68, super public clones found in 20 samples of 68, data unpublished).

In the course of the analysis, we used clone sets normalized to 25,000 cDNA events. Using a list of 7,200 public clonotypes, we compared their frequency to the frequencies in the reference cohort of healthy individuals and patients with vasculitis, to reveal that younger patients had reliably fewer public clonotypes ($p = 0.04$, Mann–Whitney U, Fig. 2, E) in their TCR repertoires, compared to healthy donors of the same age.

DISCUSSION

As mentioned above, no research works have been published so far dedicated to deep sequencing of TCR repertoires of patients with GPA and EGPA. Sanger sequencing of T-cell receptors has, however, been used to discover a common CDR3 motif among highly represented Vbeta 21 clonotypes of the TCR family in patients with EGPA [17]. Also, there are reports on the Vbeta8 clonotype dominating the TCR repertoire of patients with GPA [18].

In the course of our study, we did not observe any specific clonotypes or expression patterns of V- and J-segments that could be used to discriminate between GPA or EGPA patients and healthy individuals of the same age. Perhaps, we need a larger sample to identify T-cell clones associated with the disease. In the recent study conducted in 191 patients with ankylosing spondylitis and 227 healthy individuals, the consensus TCR beta CDR3 motif was identified to implicate in the pathogenesis of this autoimmune disease [21]. A combination of techniques, such as the analysis of TCR repertoires obtained from the lymphocytes migrating to inflammation sites and a new algorithm for calculating the probability of assembly of a particular CDR3 sequence, may provide a solution to the problem of autoimmune clone identification [22].

The analysis of TCR diversity demonstrated the absence of significant differences in the degree of clonality and the observed CDR3 diversity between patients with vasculitis and healthy controls. Slight variations in the proportion of naive T-lymphocytes and Chao1 values may indicate a minor peripheral expansion of naive clones recovered after CPH treatment.

Our study demonstrates that young patients have a significantly lower number of public clonotypes in comparison with healthy donors of the same age. Such structural changes of the TCR repertoire of young patients after exposure to high doses of CPH may indicate premature of T-cell adaptive immunity. We conclude that the subset of public TCR representing naive T-lymphocytes formed during the embryonic stage is considerably downsized by high doses of CPH. Further research is necessary to demonstrate the long-term effects of such changes.

CONCLUSIONS

Using deep sequencing, we have analyzed TCR repertoires of diseased individuals and healthy donors to discover that TCR repertoires are able to recover after the exposure to high doses of cyclophosphamide. The number of public clonotypes in patients with autoimmune disorders is, however, lower than in healthy individuals, which indicates premature ageing of TCR repertoires.

We have not observed any changes that may be associated with autoimmune vasculitis. Patients' sequencing datasets contained no T-cell clonotypes previously annotated in the literature as implicated in the pathogenesis of the disease.

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