T-CELL RECEPTOR CHAIN CENTRICITY IN THE PRIMARILY ACTIVATED EFFECTORS AND RE-STIMULATED MEMORY CELLS

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T cells, the adaptive immunity effectors, carry an antigen-recognizing T-cell receptor (TCR) that represents an αβ heterodimer. Functional dominance of one chain has been reported for a number of TCRs. This feature is called chain centricity. Today, it is unclear whether chain centricity is an inherent feature of some TCRs, and what mechanism underlies its development. The study aimed to determine the abundance of such receptors in the repertoire of primarily activated effectors and re-stimulated memory cells of mice specific to the allogeneic tumor antigens. The long-lived memory cells formed in the primary immune response *in vivo* were *in vitro* re-stimulated with the immunizing tumor cells. Primary effectors were obtained *in vitro* in the culture by stimulation of T cells of non-immunized mice with cells of the same allogeneic tumor. TCR libraries of effectors involved in the primary and secondary immune response were created by NGS sequencing. To identify chain-centric TCRs, 10 TCRα variants were selected from each repertoire. T cells of intact mice were modified with individual TCR α-chain variants by transduction, with subsequent assessment of T cell proliferation under exposure to specific allogeneic stimulators. *In vitro* screening revealed 10% of chain-centric receptors in the primary effector pool, and the proportion of such TCRs in the repertoire of re-activated memory cells was 30%. Thus, chain centricity is an inherent property of some TCRs, but secondary antigenic stimulation can be a factor for selection of clonotypes with such receptors.

Keywords: T-cell receptor, chain centricity, dominant-active α-chain, primarily activated effectors, memory T cells

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ЦЕПЕЦЕНТРИЧНОСТЬ Т-КЛЕТОЧНЫХ РЕЦЕПТОРОВ У ПЕРВИЧНО АКТИВИРОВАННЫХ ЭФФЕКТОРОВ И РЕСТИМУЛИРОВАННЫХ КЛЕТОК ПАМЯТИ

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Т-клетки, эффекторы адаптивного иммунитета, оснащены антигенраспознающим Т-клеточным рецептором (ТКР), который представляет собой αβ-гетеродимер. Для ряда ТКР было показано функциональное доминирование одной цепи, и эта особенность рецептора получила название цепецентричности. В настоящее время неизвестно, является ли цепецентричность врожденным свойством некоторых ТКР и каков механизм ее формирования. Целью работы было установить частоту встречаемости подобных рецепторов в репертуаре эффекторов и рестимулированных клеток памяти мыши, специфичных к антигенам аллогенной опухоли. Сформированные в ходе первичного иммунного ответа *in vivo* долгоживущие клетки памяти рестимулировали клетками иммунизирующей опухоли *in vitro*. Первичные эффекторы получали в культуре *in vitro* путем стимуляции Т-клеток неиммунизированных мышей клетками этой же аллогенной опухоли. Методом NGS-секвенирования были созданы библиотеки ТКР эффекторов, вовлеченных в первичный и вторичный иммунный ответ. Для идентификации цепецентрических рецепторов были отобраны по 10 вариантов ТКРα из каждого репертуара. Путем трансдукции Т-клетки интактных мышей модифицировали индивидуальными вариантами α-цепей ТКР с последующей оценкой уровня их пролиферации в присутствии специфических аллогенных стимуляторов. В ходе скрининга *in vitro* выявлено 10% цепецентрических рецепторов в пуле первичных эффекторов, при этом доля таких ТКР в репертуаре реактивированных клеток памяти составила 30%. Таким образом, цепецентричность является исходно присущим свойством некоторых ТКР, но вторичная антигенная стимуляция может быть фактором селекции клонотипов с такими рецепторами.

Ключевые слова: Т-клеточный рецептор, цепецентричность, доминантно-активная α-цепь, первично активированные эффекторы, Т-клетки памяти

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T cells are the most important adaptive immunity effectors. To ensure realization of their functions, T cells carry an antigen-recognizing T-cell receptor (TCR) involved in recognition of peptide antigens represented by self-major histocompatibility complex (MHC) molecules. TCR is a heterodimer, which consists of α- and β-chains in the predominant type of T cells. According to the classic paradigm, both TCR chains contribute equally to recognition of MHC/peptide complexes. However, it has been reported for a number of TCRs that α- or β-chain can predominate during interaction with antigen and determine the specificity of the entire receptor [1]. About a decade ago the term "TCR chain centricity" was introduced to describe this phenomenon, and TCRs showing asymmetric functional activity of the chains were named chain-centric receptors [2, 3]. This property of some TCRs can significantly simplify and increase the effectiveness of generating therapeutic T-cell products for immunotherapy of infectious diseases and cancer [1, 4].

Today, it is unclear whether chain centricity is an inherent feature of some TCRs, and what mechanism underlies its development. In the majority of studies, chain-centric TCRs were identified in the human or mouse immune repertoire [2, 3, 5–7], which may suggest that these receptors are expressed mainly by antigen-primed T cells. It has previously been shown that the naturally occurring pool of mouse memory T cells contains about 20% of chain-centric receptors [4], but their proportion in the repertoire of effectors involved in the primary immune response is still poorly understood. Clarification of this issue will help determine the important aspects of the chain-centric TCR nature: 1) whether chain centricity is an inherent property of some receptors; 2) what is the role of antigenic stimulation in the formation or selection of such TCRs.

The study aimed to determine the abundance of TCRs with the dominant-active α-chain in the repertoire of primarily activated effectors and re-stimulated memory cells of mice.

METHODS

The study involved inbred C57BL/6 (haplotype H2-K^b) female mice (body weight 18–20 g, age 6–8 weeks) obtained from the experimental biology laboratory of the Research Institute of Experimental Diagnostics and Therapy of Tumors (Blokhin National Medical Research Center of Oncology, Moscow, Russia). Animals were kept under standard conditions (20–24 °C, a 40% relative humidity, a 12-h light/dark cycle) and withdrawn from the experiment by cervical dislocation. To generate memory cells *in vivo*, nine C57BL/6 mice were immunized with P815 allogeneic mastocytoma cells (K^dD^d) via a single intraperitoneal injection of 1×10^7 tumor cells/mouse. Two months after immunization [8] the animals were withdrawn from the experiment as stated above, and the spleen was isolated under sterile conditions. Cells of the spleen were squeezed out carefully from the splenic stroma in the Potter homogenizer (DWK Life Sciences; Germany) in 3 mL of PBS. Then cytometry analysis of splenocytes was carried out in the FACSCantoII system (BD; USA) using fluorescent labeled antibodies (BioLegend; USA) to T-cell surface markers: CD3-PE, CD8-Pacific blue, CD44-APC, and CD62L-APC-Cy7. Cell debris was excluded from analysis based on the light scattering values and propidium iodide (BD; USA) incorporation. The percentage of T cells (%) in the total population of live splenic leukocytes was determined based on the CD3 marker expression. The relative number (%) of cytotoxic $CD8^+$ T cells was assessed in the $CD3^+$ lymphocyte pool. The long-lived $CD8^+$ memory T cells generated *in vivo* after immunization were determined based on co-expression of CD44 and CD62L markers (Fig. 1). Splenic cells of the P815-immunized mice were re-stimulated with the immunizing tumor antigens *in vitro* without pre-sorting [9]. For that splenocytes (4×10^5 cells/well) were seeded in triplets into 96-well round bottom plates (Corning Costar, Sigma Aldrich; USA). P815 mastocytoma cells were treated with cytostatic mitomycin C (Kyowa Hakko Kogyo Co., Ltd.; Japan) in a dose of 50 μg/mL for 60 min at 37 °C and added to splenocytes at a ratio of 1 : 10. Cells were cultured in 200 μL of the RPMI-1640 medium (PanEco; Russia) enriched with 10% fetal bovine serum (HyClone, GE Healthcare; USA), 0.01 mg/mL of ciproflaxacin (KRKA; Slovenia) and 10 μM of 2-mercaptoethanol (Merck; Germany) for 72 h at 37 °C, 5% CO_2. To induce primarily activated $CD8^+$ effectors, splenic cells of six intact (not immunized with mastocytoma P815) C57BL/6 mice were *in vitro* cultured with P815 cells for 72 h, as described above. To assess baseline proliferation, splenic cells of intact and immunized mice were similarly cultured without P815. Cell proliferation (counts per minute) in the splenocyte culture was measured based on incorporation of ^{3}H-thymidine (1 μCi/well) (Isotope; Russia) added for the last 8 h of cultivation. The antigen-induced response index was calculated as the ratio of splenocyte proliferation under exposure to P815 and corresponding baseline proliferation (Fig. 2).

The primarily activated effectors and re-stimulated memory cells obtained as described above from one intact (non-immunized) and one immunized mouse, respectively, were used to create the TCR cDNA libraries by NGS sequences on the MiSeq platform (Illumina; USA) [9].

The full-length cDNA of the TCR α-chain from each repertoire was cloned into the MigRI retroviral vector containing the PGK promoter [4]. Transfection of the HEK293T packaging cell line was performed using the calcium phosphate method. To obtain T cells transduced with an individual TCRα variant, preliminary activation of T cells of intact (non-immunized) mice was performed. For that animals were euthanized by cervical dislocation; the spleen and mesenteric lymph nodes were recovered under sterile conditions, and cells were isolated from these organs as described above. The cells obtained were then *in vitro* activated with the T-cell mitogen, concanavalin A (3 μg/mL) (Sigma Aldrich; USA), for 24 h and transduced by two spinoculations with the retroviruses containing an individual TCRα variant at 2000 × g for 2 h (22 °C) [10]. The lymphocyte modification levels were determined 48 h later by flow cytometry based on the GFP reporter protein expression measured in the control sample of T cells similarly transduced with the GFP retrovirus [10]. The transduction efficacy was 40–70% (data not shown).

T cells (1×10^5 cells/well) were seeded in triplets into the 96-well flat-bottom plates (Corning Costar, Sigma Aldrich; USA) 48 h after transduction. Cells of the EL-4 lymphoma syngeneic for C57BL/6 mice and of the immunizing mastocytoma P815 were treated with mitomycin C. To determine proliferation levels of modified T cells under exposure to syngeneic stimulators, EL-4 cells treated with the cytostatic were added to T cells at a ratio of 1 : 2. To assess specific antigen-induced proliferation, modified T cells were co-cultured with P815 cells treated with mitomycin C. Cells were cultured in 200 μL of the RPMI-1640 medium (PanEco; Russia) enriched as described above for 72 h at 37 °C, 5% CO_2. The non-transduced lymphocytes (NTLs) and GFP-modified T cells were used as controls. To assess baseline proliferation, T cells (NTLs, TCRα- and GFP-transduced) were similarly cultured without tumor cells. Cell proliferation levels were assessed using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit (Promega; USA) in accordance with

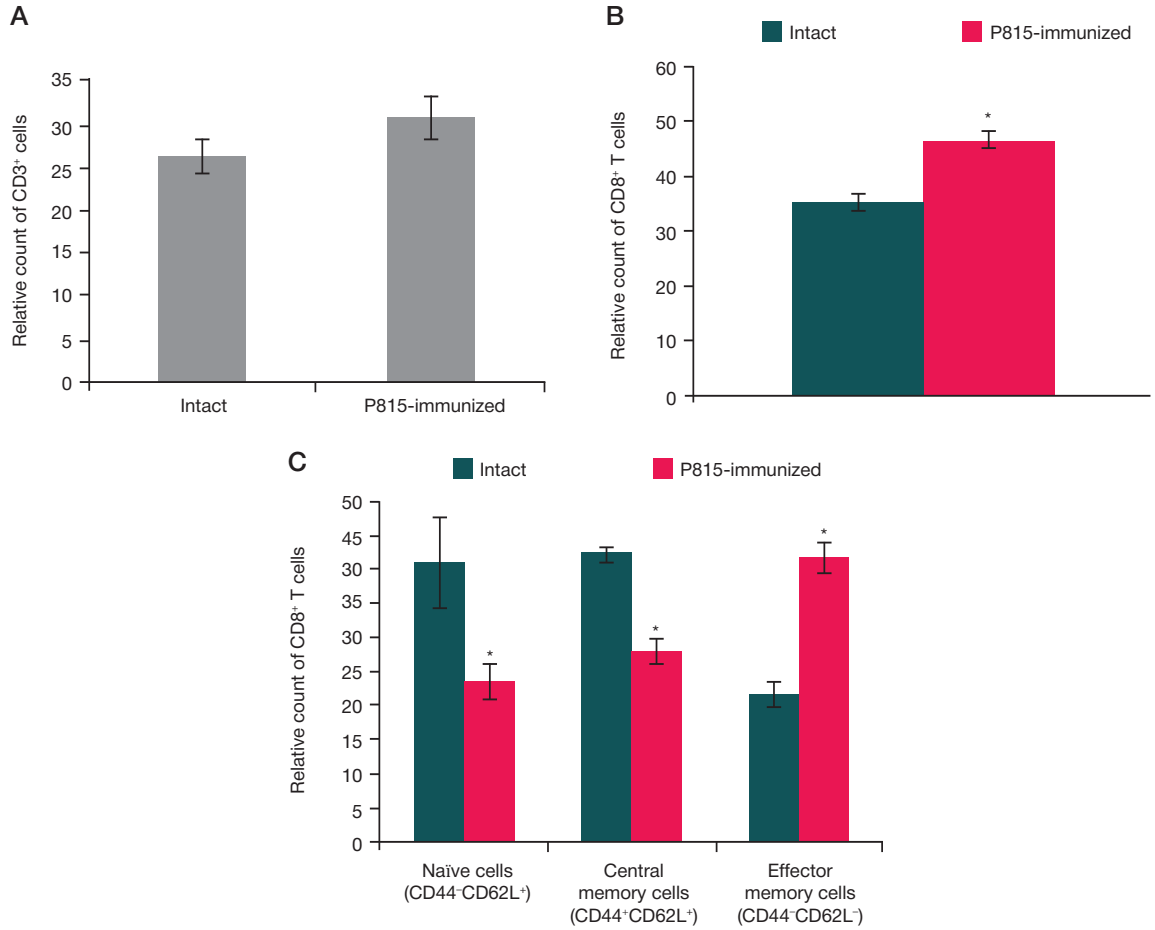

Fig. 1. Analysis of mouse splenic T cells after *in vivo* immunization with the allogeneic tumor cells. C57BL/6 mice (n = 9) were immunized intraperitoneally with allogeneic mastocytoma P815 cells. Spleen cells were analyzed by flow cytometry 2 months after immunization. Splenocytes of intact (not immunized with P815) C57BL/6 mice (n = 6) were used as a control. **A**) The relative count (%) of T cells ($CD3^+$). **B**) The percentage (%) of cytotoxic $CD8^+$ T cells. **C**) The percentage (%) of $CD8^+$ T cells with the phenotype of naïve cells ($CD44^-CD62L^+$), central memory cells ($CD44^+CD62L^+$), and effector memory cells ($CD44^+CD62L^-$). The data are presented as m ± SEM (n = 6–9). *$p \leq 0.05$ compared to the intact control (Student's t-test)

the manufacturer's instructions. Optical density (OD) was measured using the Infinite F50 microplate spectrophotometer (Tecan; Switzerland). *In vitro* screening of each TCRα variant was conducted in at least two independent experiments.

Fig. 1 and 2 present the data of three independent experiments as mean ± standard error of the mean (mean ± SEM) (n = 6–9). Fig. 3 presents frequencies of unique TCRα clonotypes in each studied repertoire. Fig. 4 presents the data of one of the two representative experiments as mean ± SEM for three technical replicates. Statistical analysis was performed using the unpaired Student's t-test after testing the sample distribution for normality using the Kolmogorov–Smirnov test. The differences were considered significant at $p < 0.05$. Statistical analysis was performed using the Prism v.8.1.2 software (GraphPad; USA).

RESULTS

In this study, the experimental model of generating long-lived memory T cells was used involving *in vivo* immunization of C57BL/6 mice ($H2\text{-}K^b$) with P815 allogeneic mastocytoma cells (K^dD^d). Due to allogeneic differences in MHC class I molecules, the recipient develops predominantly $CD8^+$ T-cell response to the transplanted tumor. No increase in the relative number of T cells in the spleen of immunized animals compared to intact mice was reported (Fig. 1A), however, the proportion of $CD8^+$ T cells after immunization was significantly higher relative to the control (Fig. 1B). Furthermore, in the spleen of immunized mice, accumulation of $CD8^+$ T cells with the phenotype of effector memory cells ($CD44^+CD62L^-$) was reported, the percentage of which increased 1.9-fold relative to the same population of $CD8^+$ T cells in the spleen of intact mice (Fig. 1C). The data obtained suggest that a pool of long-lived $CD8^+$ memory cells is formed *in vivo* after contraction of primary immune response.

Considering the fact that the T cell surface activation phenotype is not directly correlated with the T cell experience of interaction with antigen and its functional status [11], splenic lymphocytes of the immunized mice were re-stimulated with the immunizing tumor antigens *in vitro* in order to confirm generation of true memory T cells during *in vivo* immunization. Primary proliferative response of splenocytes of intact mice was obtained *in vitro* in the culture with P815 allogeneic mastocytoma cells, which was twice higher compared to baseline proliferation values (Fig. 2). Furthermore, the antigen-induced proliferation level of splenocytes from the immunized animals was three times higher compared to that in the cell culture of intact mice (Fig. 2). Thus, the *in vitro* functional test showed the primary immune response to the allogeneic tumor cells and the enhanced secondary response of memory T cells.

This experimental system was used to generate effectors of the primary and secondary immune response to the allogeneic tumor cells and construct libraries of their TCR α-chains (TCRα). To identify TCRα clonotypes involved in the immune response to the P815 antigens, the repertoires of primarily activated effectors and re-stimulated memory cells were compared to the TCRα repertoires of non-immunized mice and immunized mice without antigenic stimulation *in vitro*, respectively. TCRα

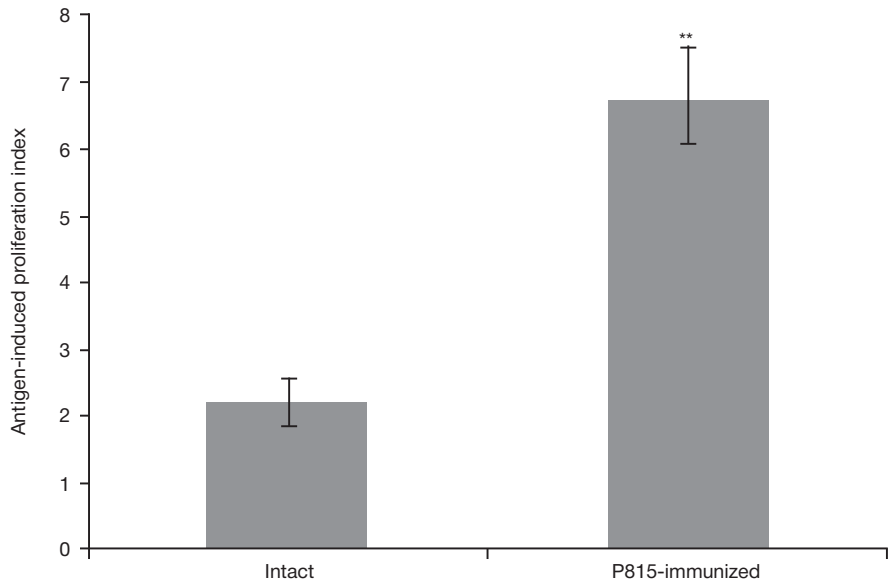

Fig. 2. Levels of antigen-induced proliferation *in vitro* of T-cells in the primary and secondary immune responses to the allogeneic tumor cells. Splenic cells of intact (not immunized with P815) and P815-immunized C57BL/6 mice were cultured in the presence of allogeneic mastocytoma P815 for 72 h. Splenocytes similarly cultured without antigenic stimulation were used to assess baseline proliferation. The index of antigen-induced proliferative response to alloantigens was calculated as described in the Methods section. The data are presented as m ± SEM (n = 6–9). **$p \leq 0.01$ (Student's t-test)

variants, the frequency of which after stimulation was at least three times higher compared to frequency of the same clonotype in the corresponding repertoire without antigenic stimulation, were determined in each library [9]. A total of 10 TCRα variants unique for the repertoire of primarily activated effectors or re-stimulated memory cells were selected for further research (Fig. 3).

The dominant-active TCRα were identified in an *in vitro* test system. For that T cells of the intact mice modified with each TCRα variant were introduced to the culture with syngeneic stimulators (EL-4 cells) or specific allogeneic stimulators (P815 cells) (Fig. 4). The appropriate α-chain was considered to be dominant-active, when the proliferation level of transduced lymphocytes under exposure to P815 was significantly higher, than the proliferation level of the same cells not subjected to antigenic stimulation (baseline), in the presence of EL-4 (stimulation with the syngeneic tumor), or when it was significantly higher, than the values of antigen-induced proliferation of NTLs and GFP-modified T cells (Fig. 4).

The screening revealed one dominant-active TCRα from the repertoire of primarily activated effectors (#3; Fig. 4A): the level of proliferative response of T cells modified with the α-chain TCR #3 in the presence of the specific allogeneic stimulator (P815 cells) was 1.3-fold ($p < 0.05$) higher, than the level of their proliferation in the culture with syngeneic stimulators (EL-4 cells). Three dominant-active TCRα variants were identified in the repertoire of re-activated memory cells: T cells transduced with TCRα #2, TCRα #5, and TCRα #9 had significantly 1.3-fold increased ($p < 0.05$) proliferation activity under specific antigenic stimulation with P815 cells compared to their proliferation in the presence of syngeneic EL-4 cells (Fig. 4B).

Thus, the study has shown that the primarily activated effector repertoire comprises 10% of chain-centric TCRs, while the proportion of such receptors in the repertoire of re-stimulated memory cells is 30%. This preliminary assessment is currently being refined with additional data.

In vitro screening also revealed distinct variants of TCR α-chains (#9 in the primarily activated effector repertoire (Fig. 4A) and #1 in the repertoire of re-stimulated memory T cells (Fig. 4B)), modification with which resulted in enhanced T cell proliferation in the presence of syngeneic EL-4. This may be explained by the generation of a receptor with the new specificity as a result of the interaction of the transduced α–chain with an endogenous TCR β-chain in a mature T cell.

DISCUSSION

Using the previously developed experimental model we traced sequential changes in the mouse TCR repertoire during the immune response to tumor alloantigens, from the primary response to immunological memory formation and induction of the secondary memory cell response [8, 9]. The data obtained have shown that both primarily activated effectors and re-stimulated memory T cells express TCRs with the dominant-active α-chain. Based on this finding, it can be assumed that chain centricity is an inherent property of some TCRs.

An interesting analogy for this phenomenon can be found in the paper by Dietrich et al. reporting the study of the pre-immune repertoire of T cells specific for the melanoma-associated autoantigen melan-A. It has been shown that melan-A-specific thymocytes and mature peripheral T cells preferentially use a particular V segment of the α-chain (Vα 2.1) [12]. Thus, narrowing of the repertoire in favor of using this α-chain variant occurs during intrathymic selection of melan-A-specific T cells, although the fact that they preferentially use this TCRα variant does not indicate the α-chain functional dominance. We believe that the reasons for this phenomenon can include the possibility of repeated rearrangements of the TCR α-chain genes during positive selection in the thymus, resulting in the selection of their variants capable of establishing multiple contacts with the endogenous MHC/peptide complexes and, therefore, of more effective positive selection.

It should be noted that in our experiments the proportion of chain-centric receptors in the repertoire of re-activated memory cells was higher (30% vs. 10% in the primarily activated effector repertoire), which can suggest selection of the TCR clonotypes with the dominant-active α-chains during secondary specific antigenic stimulation. Bioinformatics analysis of the entire TCR repertoire of these two functional groups has shown that physicochemical characteristics of TCRα in the pool of re-activated memory cells markedly differed from the properties of the TCR α-chains of effectors involved in the primary immune

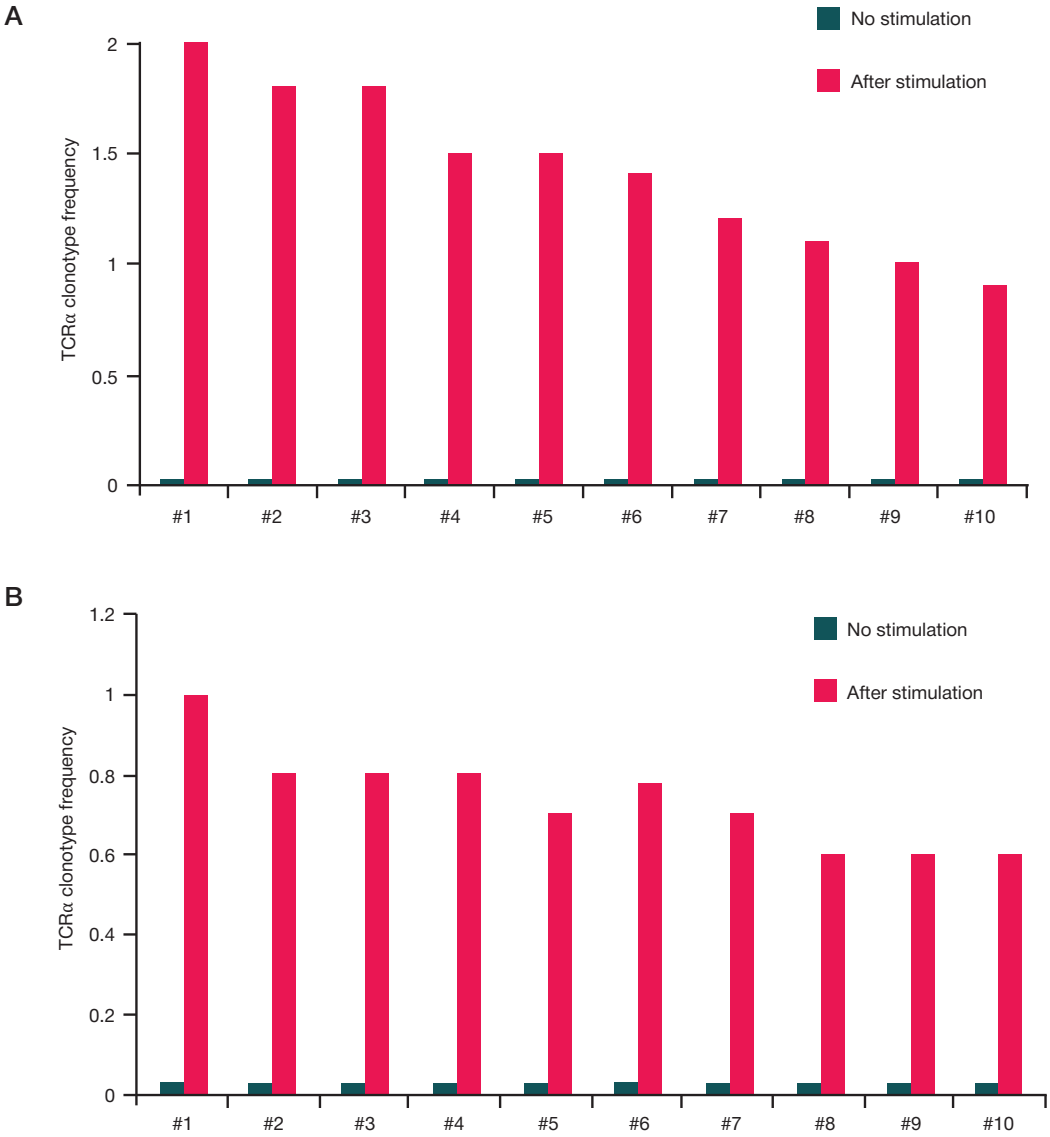

Fig. 3. T-cell receptor α-chain variants selected for *in vitro* screening. Frequency of the T-cell receptor α-chain clonotype in the repertoire of effectors involved in the primary immune response **(A)** and of re-stimulated memory T cells **(B)**

response [9]. According to the data obtained, in the secondary response, the TCR repertoire is enriched with receptors containing the α-chain with the increased strength of binding to the MHC/peptide complexes and cross-reactivity [9].

It is well known that TCRs of memory cells have the increased affinity for antigen [13, 14]. The results of our studies suggest that this can be also associated with the expression of the dominant-active TCRα. Thus, selection of chain-centric TCRs can represent one of the mechanisms underlying maturation of functional avidity of antigen-primed T cells [15–17].

In the light of the currently available data, we believe that the functionally true memory T cells can be the most promising source of therapeutic TCRs. It has been previously shown in the *in vivo* experimental models that the dominant-active α-chains of the memory cell chain-centric TCRs can be successfully used to generate T-cell products for adoptive immunotherapy of cancer and infectious diseases [4, 7].

The *in vitro* screening system described in this study can be also used to assess possible autoreactivity of modified T cells. During transduction of an individual TCR α-chain into T cells, the α-chain binds to endogenously rearranged β-chains, and this can result in the generation of receptors with new specificity, including potentially autoreactive TCR. Thus, in our study we have revealed increased proliferative activity of T cells modified with two TCRα variants in the presence of syngeneic stimulators (Fig. 4A, #9; Fig. 4B, #1). However, determination of autoreactivity of these transduced T cells was outside the scope of this study. Meanwhile, we have earlier shown that T cells modified with the dominant-active TCRα do not show nonspecific cytotoxicity when adoptively transferred into syngeneic recipients, which confirms the lack or low rate of potentially autoreactive clones in the resulting T cell product [18].

CONCLUSIONS

In this study we developed an *in vitro* system for screening of mouse chain-centric TCRs. Using this system, the dominant-active antigen-specific α-chains were identified both in the repertoire of effectors involved in the primary immune response and in the repertoire of memory cells after the secondary specific antigenic stimulation. The study results have shown that 10% of the TCRs of primarily activated effectors are chain centric. Thus, this property is inherent to some T-cell receptors. Furthermore, in the secondary immune response, the proportion of such receptors increases 3-fold, which suggests that the repertoire is enriched with chain-centric TCRs due to antigen-induced

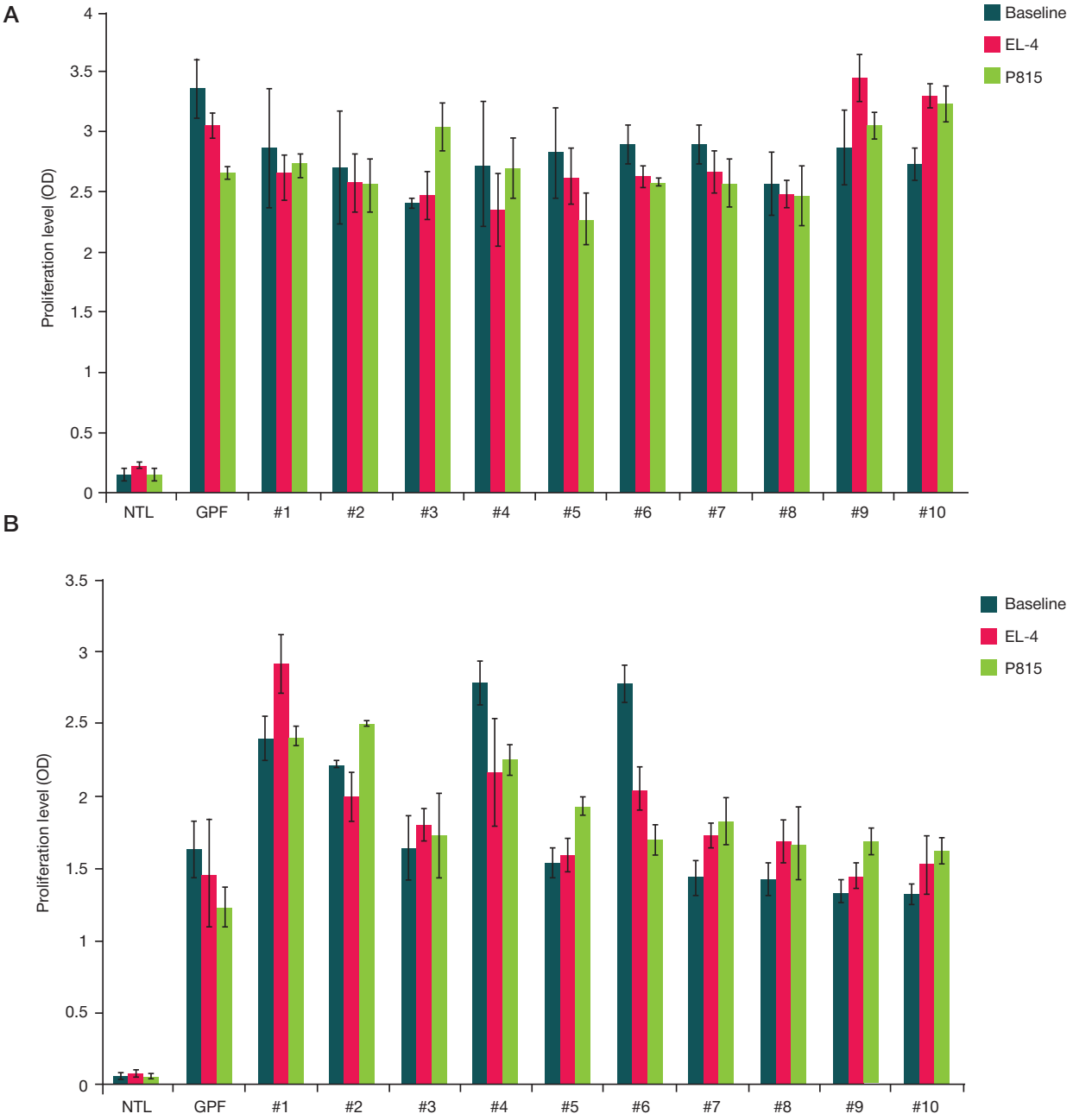

Fig. 4. *In vitro* antigen-induced proliferation levels of T cells transduced with individual T-cell receptor (TCR) α-chain variants. Activated T cells of an intact C57BL/6 mouse were transduced with the TCR α-chains from the repertoire of primarily activated effectors **(A)** or re-stimulated memory cells **(B)**. Modified lymphocytes were placed in the culture with syngeneic stimulators (EL-4 lymphoma cells) or allogeneic specific stimulators (P815 mastocytoma cells) for 72 h. To assess baseline proliferation levels, transduced T cells were cultured without tumor cells (baseline). Non-transduced activated lymphocytes (NTLs) and GFP-modified T cells were used as controls. The data of one representative experiment out of two are presented as m ± SEM for three technical replicates. *$p \leq 0.05$ (Student's *t*-test)

selection of clonotypes with such receptors. The findings will help to improve the process of identifying and selecting chain-centric antigen-specific TCRs, which are a promising source of therapeutic receptors for adoptive immunotherapy.

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