TESTING OF MONOCLONAL ANTIBODIES AGAINST THE T-CELL RECEPTOR ASSOCIATED WITH ANKYLOSING SPONDYLITIS

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In the last decade there has been a tendency to move away from the symptomatic treatment and embrace targeted therapies. This process is underpinned by the accumulated knowledge of the mechanisms underlying the pathogenesis of diseases and driven by the advances in biotechnologies. T-cell receptors with variable TRBV9 \(\beta\)-chain regions have been recently associated with spondyloarthritides including its subtype, ankylosing spondylitis. The aim of this work was to engineer a chimeric monoclonal antibody targeting the variable region of the T-cell receptor \(\beta\)-chain encoded by the TRBV9 gene segment and assess its specificity and cytotoxicity. Using flow cytometry and next generation sequencing, we demonstrate that the engineered chimeric antibody is highly specific and exhibits cytotoxic activity against its target. Approaches based on the use of therapeutic chimeric antibodies against pathogenic T-clones may hold great promise for the therapy of autoimmune disorders in general and AS in particular.

Keywords: autoimmune disease, ankylosing spondylitis, therapeutic antibody for autoimmune treatment, T-cell receptor

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ТЕСТИРОВАНИЕ МОНОКЛОНАЛЬНЫХ АНТИТЕЛ К Т-КЛЕТОЧНОМУ РЕЦЕПТОРУ, АССОЦИРОВАННОМУ С АНКИЛОЗИРУЮЩИМ СПОНДИЛИТОМ

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В последние десятилетия в лечении аутоиммунных заболеваний прослеживается тенденция к замещению симптоматической на молекулярно-таргетную терапию. Предпосылками для этого служат как установленные механизмы развития заболевания, так и прогресс в области биотехнологии. Недавно было показано, что T-клеточные рецепторы с вариабельными участками \(\beta\)-цепи TRBV9 ассоциированы с спондилоартропатиями, включая анизлозирующий спондилиит. Целью данной работы было получить, определить специфичность и оценить цитотоксичность химерного моноклонального антитела, взаимодействующего с вариабельным участком \(\beta\)-цепи Т-клеточного рецептора, который кодируется генетическим сегментом TRBV9. С помощью цитометрического анализа, а также массированного секвенирования показано, что химерное антитело обладает высокой специфичностью и цитотоксической активностью. Получение лечебного антитела к потенциально патогенному Т-клону может быть перспективным подходом для терапии аутоиммунных заболеваний в целом и АС в частности.

Ключевые слова: аутоиммune заболевание, анизлозирующий спондилиит, терапевтические антитела, лечение аутоиммунных заболеваний, T-клеточный рецептор

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Ankylosing spondylitis (AS, or Bekttherve’s disease) is a chronic progressive autoimmune disease associated with HLA B2705 and characterized by the inflammation of the spine and sacroiliac, intervertebral and costovertebral joints that causes stiffness and eventually leads to spinal fusion. Treatment options include physical therapy and anti-inflammatoryatories, mostly nonsteroidal, such as ibuprofen, diclofenac, or indomethacin. The use of steroids in patients with AS is only scarcely covered in the literature. Some authors report that high doses of prednisolone (50 mg a day) administered over the course of two weeks provide a good short-term fix [1]. Others describe a sustained symptomatic relief observed for an entire year following the TNF therapy [7]. The injection of high doses of corticosteroids [2]. However, steroids also cause their typical side effects, including high blood pressure, elevated blood glucose and reduced bone mineral density. Patients with severe AS are prescribed phenylbutazone and opioids to ameliorate pain. The existing regimens bring partial relief but cannot prevent the disease from progressing and do not protect against ankylosis while causing complications, such as digestive tract problems, etc. [3].

The real breakthrough in the therapy of AS made in the last 15 years is linked to the use of biologic inhibitors of tumor necrosis factor alpha (TNF-α), among which are the monoclonal antibodies infliximab, adalimumab, golimumab, and certolizumab pegol. TNF is the major mediator of inflammation also involved in the progression of other autoimmune disorders [4]. In 2010 TNF inhibitors were added to the list of AS therapies recommended by the Assessment of Spondyloarthritis International Society (ASAS) [5]. Thus, infliximab suppresses inflammation and therefore alleviates pain and reduces stiffness [6]. However, clinical data show that only 50–60% of patients with AS respond to anti-TNF therapy [7]. Besides, although inhibition of TNF or its receptor reduces inflammation, it cannot stop the progression of the disease [8, 9].

It is known that autoimmune disorders are accompanied by overproduction of IL17, in which a few different cell types are involved, including CD4+ T helpers, gamma delta and CD8- and CD8- negative selections occurring in the thymus. Autoaggressive T cells are eliminated. Pathologic T-cell autoreactivity is a cause of many autoimmune conditions.

One of the approaches to the treatment of autoimmune diseases discussed in the literature is based on the use of antibodies that recognize all alpha/beta TCRs. It is described in the work [18] demonstrating that administration of an anti-TCR antibody right after the injection of myelin peptide MOG35-55 inducing experimental autoimmune encephalomyelitis (EAE) blocks the development of this condition. The authors did observe the predicted depletion of CD4+ and CD8+ cells, but these T-cell subsets behaved differently: CD4+ were the first to deplete and recover, while in CD8+ both processes were delayed. The authors opened the way to the therapeutic effect of the treatment: in contrast to the CD3-based therapy, no signs of the disease were observed even after the T-cell population was completely restored. Although both therapies aim to deplete T cells, the intracellular pathways activated by the TCR-CD3-antibody complex are different and produce different effects. Monoclonal antibodies targeting variable domains typical for autoreactive TCRs seem to be a promising therapeutic tool. They cause selective elimination of T-cell subsets with pathogenic clones. The arrival of NGS technologies has given rise to new approaches based on the deep sequencing of TCR repertoires. The difficulty of such approaches lies in the great diversity of TCRs [19]. Identification of pathogenic clones is based on the comparison of TCR repertoires of healthy and diseased individuals and therefore becomes a very labor-consuming task. It is also important to account for the structural features of proteins constituting the major histocompatibility complex involved in antigen presentation as these features also affect the composition of a T-cell repertoire.

In spite of the obstacles, the T-cell clones potentially implicated in the pathogenesis of AS and the CD3R consensus motif were finally identified in 2017 [20, 21]. The pathogenic clones were present in the synovial fluid and peripheral blood of patients with AS. Interestingly, the pathogenic TCRs were not detected in the samples of healthy individuals at the same sequencing depth regardless of whether the participants had the HLA*B27 allele or not. The identified AS-associated TCRs carried the gene segment TRBV9 (name specified in the IMGT database) coding for the variable domain of the β-chain. The obtained data suggest that anti-TRBV9 antibodies have a therapeutic potential for treating AS.

Elimination of T cells carrying a pathogenic TCR is an indispensable prerequisite for the effective therapy of autoimmune diseases. It is known that IgG1 antibodies, which have an Fc-region, induce death of the target cell by binding to it. There are two possible explanations of this phenomenon: antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC the antibody with a particular Fc binds its epitope on the surface of the target cell. An effector T cell that expresses an FcR receptor or a CD16 molecule recognizes the Fc domain and binds to it. Formation of this triple complex triggers a cascade of reactions inside the effector T cell resulting in the release of cytotoxic granules. Thus, the death of the target cell is mediated by the perforin-granzyme pathway. The effectiveness of Fc binding to the receptor of the effector T cell depends on the immunoglobulin allotype and the pattern of amino acid glycosylation. Therefore, the cytotoxic activity of the antibody can vary [22]. CDC is similar to ASCC but in CDC a complement complex is assembled on the cell surface triggering a cascade of reactions that eventually induce apoptosis of the target cell. The therapeutic antibodies modelled for the present work carried the same IgG1 Fc allotype as rituximab.
The aim of our study was to assess specificity and cytotoxicity of a few variants of engineered monoclonal antibodies against the variable TRBV9 domain of the TCR β-chain in vitro.

METHODS

Blood donors

Samples of peripheral blood were collected from two 53-year-old male donors 7 times at a minimum interval of 7 days between the procedures. The study was approved by the Ethics Committee of Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology (Protocol 2013–5/4).

Isolation of mononuclear cells from peripheral blood

Peripheral blood samples were collected into K2-EDTA-containing Vacutte tubes (4 ml of blood per tube) and diluted fourfold with PBS. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-urografin density gradient centrifugation (density of 1.077 g/cm³) (PanEco; Russia). Briefly, the diluted blood sample was layered onto the Ficoll-urografin solution at the ratio of 1:1. Then, the sample was centrifuged in the swinging bucket rotor at room temperature and 400 g for 30 min. The cell suspension was collected from the interface, washed twice in PBS, and centrifuged again at room temperature and 400 g for 10 min. PMBC count was done in the Goryaev chamber.

Chimeric antibodies and their kinetic screening by biolayer interferometry

Monoclonal antibodies MA-K1, MA-K2, MA-K3, and MA-K4 were manufactured by the biotech company Biocad and their nucleotide and amino acid sequences were published in the patent application. These antibodies contain chimeric heavy- (H) and light- (L) chains with a variable region of the rat immunoglobulin and a constant region of the human immunoglobulin. The degree of antibody humanization is 65%.

To measure the dissociation constant of the TCR/AB complex, we performed biolayer interferometry using the ForteBio Octet RED384 detection system (Pall Corporation; USA). The soluble TCR taken at a concentration of 20 μg/ml was immobilized on the surface of AR2G sensors (ForteBio) in the buffer was recorded for 600 sec. Reference subtraction and the 1:1 Global interaction model.

Interaction of the studied МА-К1, МА-К2, МА-К3, and МА-К4 antibodies with different TCR complexes. The table features the dissociation rates and dissociation constants of the formed complexes. The measurements were done using ForteBio Octet RED384.

| Table | Interaction of the studied MA-K1, MA-K2, MA-K3, and MA-K4 antibodies with different TCR complexes. The table features the dissociation rates and dissociation constants of the formed complexes. The measurements were done using ForteBio Octet RED384. |
|-------|---------------------------------------------------------------------------------------------------------------|
|       | TRBV9 + TRAV26  | TRBV9 + TRAV38  | TRBV7 + TRAV38 |
| Kd    | Kdis  | Kd    | Kdis  | Kd    | Kdis  |
| MA-K1 | 2.9E-10 | 1.79E-04 | 3.21E-10 | 2.26E-04 | Does not interact |
| MA-K2 | <1.0E-12 | <1.0E-07 | <1.0E-12 | <1.0E-07 | Does not interact |
| MA-K3 | 2.44E-10 | 1.63E-04 | 3.53E-10 | 1.23E-04 | Does not interact |
| MA-K4 | <1.0E-12 | <1.0E-07 | <1.0E-12 | <1.0E-07 | Does not interact |

Flow cytometry analysis

To visualize their reactivity, the studied antibodies MA-K1, MA-K2, MA-K3, and MA-K4 were labeled with fluorescein isothiocyanate (Sigma; USA) following the manufacturer’s protocol. The number of fluorochromes that reacted with antibodies was evaluated based on the ratio of the absorption spectra at 495/280 nm wavelengths.

A total of 10° PMBC cells separated by Ficoll-urografin density gradient centrifugation as described above were incubated with each of the FITC-labeled monoclonal antibodies MA-K1, MA-K2, MA-K3, and MA-K4 taken at two different concentrations (5 μg/ml (A) and 200 ng/ml (B)) and CD3-eFlour450 (clone UCHT1; eBioscience; USA) added in the quantity recommended by the manufacturer. The sample was transferred to 50 μl 1X PBS supplemented with 0.5% BSA, incubated at room temperature for 20 min, and then washed in the same buffer solution.

Cell sorting and sequencing

PMBC (3×10⁷) were combined with 100 μl of 0.5% PBS/BSA solution, 6 μl of anti-CD3-eFlour 450 (clone UCHT1; eBioscience; USA) and each of the studied FITC-labeled antibodies (one antibody variant per sample) taken at a final concentration of 100 ng/ml. The mixture was incubated at room temperature for 20 min and washed in 0.5% PBS/BSA solution.

Cell sorting and the analysis of cell subpopulations were carried out on FACSAriaII (BD; USA). To exclude debris and cells that did not meet the criteria for the size and granularity of live lymphocytes, forward and side scatter gating was applied. Two-parameter density plots were constructed to distinguish the populations of CD3+TRBV9+ and CD3-TRBV9 cells.

To be sure of high sorting quality, we re-sorted the CD3+TRBV9+ subpopulation. The sorted cell population was 95% enriched in the target cells. The sorted cells were transferred to the RLT buffer (Qiagen; Germany) prior to RNA isolation. Total RNA was isolated using the Qiagen RNAeasy mini kit #217004 according to the manufacturer's protocol.

cDNA was synthesized from the obtained RNA template and the TCR beta chain fragment was amplified as described in [23]. PCR products were ligated to Illumina adaptors and then sequenced on MiSeq Illumina (Illumina; USA). Sequencing yield was analyzed in MiGEC, MIXCR and VDJTools [24] as described in [25]. Statistical processing was done in Graphpad Prism 3.0.

Cytotoxicity testing

The studied chimeric antibodies were tested for their cytotoxicity towards PMBC obtained from two donors. Samples of peripheral blood were collected into Vacutte K2-EDTA-containing tubes. Mononuclear cells were isolated as described above.

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The mononuclear cell fraction was transferred to PBS supplemented with 0.5% BSA and 2 mM EDTA. The total cell number was inferred from the cell count in the aliquot. Cell viability was determined by Trypan Blue staining. To evaluate the cytotoxicity of the studied antibodies, 3–4 x 10^6 cells were incubated with the MA-K2 antibody taken at different concentrations (1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μg/ml) for one hour. Following the incubation, the cells were washed twice in PBS, transferred to the RPMI media supplemented with 10% human serum (BioIVT; UK) and incubated for 24 h in the CO₂ incubator. Then the cells were collected and stained with CD4-PE (clone RPA-T4; BD Bioscience; USA), CD8-FITC (clone SK3; eBioscience; USA), CD3-eFluor450 (clone UCHT1; eBioscience; USA), and TO-Pro3readyflow (ThermoFisher; USA) as described in the manufacturer’s protocol. Staining was followed by the flow cytometry analysis on the FacsAriaIII cell sorter (BD; USA).

Fig. 1. Two-parameter density plots showing the distribution of mononuclear blood cells stained with anti-CD3-eFluor405 antibodies and 4 variants of FITC-labeled anti-TRBV9 antibodies (MA-K1, MA-K2, MA-K3, and MA-K4). Every tested anti-TRBV9 antibody was taken at two different concentrations: 3 μg/ml (A) or 200 ng/ml (B) per 10^6 mononuclear cells. The CD3*TRBV9* population is marked by a small square. The proportion of TRBV9*CD3* cells is given relative to all CD3* lymphocytes. C. Staining with different concentrations of the chimeric MA-K2 antibody: 2 ng/ml, 20 ng/ml, 50 ng/ml, 200 ng/ml (top to bottom).
RESULTS

Chimeric monoclonal antibodies MA-K1, MA-K2, MA-K3, and MA-K4 specifically bind the soluble TCRs carrying TRBV9.

In this work we tested 4 variants of the monoclonal anti-TRBV9 antibody (MA-K1, MA-K2, MA-K3, and MA-K4) different in the amino acid sequence of their hypervariable CDR3 domain.

The dissociation and binding constants of the complexes formed by soluble TCRs and chimeric MA-K1, MA-K2, MA-K3, and MA-K4 were measured by biolayer interferometry (ForteBio; Pall Corporation; USA) and surface plasmon resonance (SPR) (see the Table). Each of the studied immunoglobulins exhibited high specificity and an ability to effectively bind the target TCR encoded by the TRBV9 gene segment; the antibodies did not react with TCRs that carried a variable domain encoded by a different gene segment (TRBV7). Specificity of the studied antibodies did not depend on the composition of the TCR alpha-chain in the complex. We tested two TCR complexes carrying TRBV9 and TRAV26/TRA238 only to reveal no difference in the binding effectiveness. Based on the results of our experiments, we selected two antibody variants MA-K2 and MA-K4 for which dissociation constants and rates were the lowest (kd <1.0E-12 and kdis <1.0E-07 1/s) (see the Table).

Affinity of cytotoxic antibodies often determines their in vivo effective concentrations, specificity and safety. The chimeric MA-K2 and MA-K4 bound at high affinity to their targets, which speaks in favor of their potential use as therapeutic agents.

Chimeric immunoglobulins specifically target the population of TRBV9+ lymphocytes

As part of the flow cytometry analysis, the obtained chimeric antigens MA-K1, MA-K2, MA-K3, and MA-K4 were conjugated to fluorescein isothiocyanate. Importantly, direct labeling significantly increases the informative value of the analysis.

The studied chimeric MA taken at a concentration of 3 μg/ml stained about 3% of the CD3+ subpopulation (Fig. 1A). At 200 ng/ml (Fig. 1B) it became clear that 3 μg/ml exceed the critical concentration; at this concentration the proportion of the
stained TRBV9+ cells remained the same, but the percentage of nonspecifically bound CD3 cells increased (13 ± 2%) (Fig. 1).

Based on the maximum ratio of CD3-TRBV9+ to TRBV9-CD3 and the high intensity of the fluorescence signal indicating specific binding, we chose the MA-K2 antibody as the best candidate for further experiments. To determine the optimal antibody concentration, titration was performed at concentrations ranging from 200 to 2 ng/ml (Fig. 1C). For titration, PBMC of the healthy donor were used. The test determined the minimal MA-K2 concentration of 50 ng/ml applied to stain 106 PBMC at which the TRBV9+ cells amounted to 3% of all of the entire CD3+ population and nonspecific binding was not observed.

MA-K2 specificity was confirmed by lymphocyte sorting and the subsequent sequencing-based analysis of the TCR repertoire (Fig. 2A). For sorting, we used PBMC isolated from the peripheral blood of the healthy donor and stained with fluorescently labeled antibodies CD45, CD3 and MA-K2 taken at concentrations of 100 ng/ml each (Fig. 2A). To analyze the repertoire of T cell receptors, we selected two populations of CD3-TRBV9+ cells, as well as a population of TRBV9-CD3+ cells. Sorting was carried out in two replicates, which was necessary to demonstrate its quality. Generally, when MA-K2 antibodies specifically bind to the TCRs representing the CD3-TRBV9+ subpopulation, the corresponding cDNA library is expected to be enriched in TRBV9 transcripts, whereas the CD3-TRBV9- subpopulation is supposed to be free of the target sequences. Total RNA was isolated from all selected cell subpopulations and cDNA was synthesized using primers complementary to the constant region of the TCR beta chain. The libraries were amplified and sequenced by NGS (MiSEQ; Illumina; USA). The analysis of the obtained TCR beta chain repertoires revealed that the libraries obtained from the sorted MA-K2-stained cells were 93% enriched in the sequences encoded by the TRBV9 gene segment, whereas no TRBV9 sequences were observed in the repertoires of CD3+TRBV9+ beta chains.

This suggests high specificity and efficacy of the studied chimeric antibody.

Cytotoxicity of chimeric immunoglobulins

To assess the cytotoxic activity of the studied monoclonal antibody, we had to resort to a non-standard approach for a few reasons. First, the target population of cells constituted only a small proportion of the total cell pool (2.5% of CD3+ lymphocytes). Second, this population can be distinguished from the rest of the cells based on only one surface marker: the T cell receptor that has a unique variable beta chain domain. This obstructs the use of cell sorting for the purpose of enriching the target TRBV9+ population and complicates the classic cytotoxic test protocol (the target cell population + natural killers taken at various proportions).

Therefore, flow cytometry was employed to assess the cytotoxic activity of MA-K2. We used the mononuclear cell fraction as it contains both the target population and other cells that mediate the cytotoxic reaction (natural killers, etc.). The half maximal effective concentration (EC50) of the MA-K2 antibodies was determined in a series of in vitro experiments. The cytotoxic effect was measured based on the progressively declining proportion of TRBV9+ cells in the population of CD3+ lymphocytes correlated with an increase in MA-K2 concentrations (Fig. 2B). At a concentration of 100 ng/ml complete elimination of TRBV9+CD3+ was observed. Thus, EC50 for MA-K2 was 7 ng/ml (Fig. 2C).

To measure the proportion of dead cells among the populations of CD4+CD3+ and CD8+CD3+ lymphocytes, we added TO-Pro3readyflow (ThermoFisher) to the cells incubated with MA-K2 (Fig. 2D). Twenty-four hours after incubation with MA-K2 taken at a concentration of 100 ng/ml, a significant increase in the proportion of dead cells was observed in comparison with the control (Fig. 2D). This experiment was conducted in 7 replicates, and every time we observed an increase in the proportion of dead cells depending on the concentration of MA-K2.

To sum up, the chimeric MA-K2 antibody exhibits high cytotoxic activity and target specificity in vitro.

DISCUSSION

The existing therapeutic antibodies are successfully used to manage severe disorders, such as multiple sclerosis, some cancers and retinal degeneration. Their development relies on the knowledge of targets they are expected to work against implicated in the pathogenesis of a disease. In a recent work published by our colleagues a correlation has been shown between AS and the T-cell clones carrying a variable beta chain region encoded by the TRBV9 gene segment [20, 21]. Involvement of certain T clones in the pathogenesis of AS is yet to be confirmed, but the T cell receptor itself is a promising candidate target for a therapeutic antibody.

The engineered cytotoxic chimeric (human-rat) antibody MA-K2 is the most important outcome of our work. It specifically and effectively binds the TCR beta chain region encoded by TRBV9. To test its cytotoxicity, we used flow cytometry and TO-Pro3readyflow staining (ThermoFisher; USA), which enabled us to accurately separate dead and viable cells. This is not a common approach to assessing antibody-dependent cell-mediated cytotoxicity (ADCC). The literature describes a few methods of quantifying cells killed through an interaction with a cytotoxic antibody, but they all have their downsides. For example, the widely used method based on the detection of 51Cr [26] released in the course of cell lysis is not sensitive enough and cannot be employed to count dead cells in a cell population. This limits its application in cases when the target cell subset makes up only 3% or less of the total population. The approach exploited in this study was recently used to assess the toxicity of Trastuzumab detecting small proportions of dead cells (> 10%) in the cell culture and PBMC [27].

We hope that the engineered MA-K2 antibody has a good potential to be used in vivo to deplete the T-cell population associated with ankylosing spondylitis, thereby alleviating its symptoms. As noted previously, elimination of pathogenic clones is a promising approach to treating autoimmune diseases and another step towards precision medicine. The literature reports successful use of monoclonal antibodies against Vβ and for treating autoimmune diseases in model systems. In an experiment conducted in mice, all autoreactive T cell clones isolated from the animals with induced autoimmune encephalomyelitis carried a TCR domain encoded by the Vβ8 segment (TRBV13). Encephalomyelitis was induced by the injection of the MBP peptide. The subsequent injection of the antibody specific to that domain had a protective effect and blocked the development of the disease [28]. Another monoclonal antibody against Vβ8, KJ16, was used to protect mice against collagen-induced arthritis. Injections of this antibody significantly reduced the incidence of the condition [29].

Importantly, the proportion of cells carrying a TRBV9-encoded TCR on their surface is low and does not exceed
3%. This leads us to hypothesize that once an anti-TRBV9 is administered, no severe toxic effect will be observed caused by massive cell death and a cytokine storm, as is the case with some anti-CD3 monoclonal antibodies [30].

At present, targeted therapies rely on the use of monoclonal antibodies with different degree of humanization required to attenuate the immunogenicity of the drug. Three main types of therapeutic antibodies can be distinguished: chimeric (a constant domain of humans + a mouse variable domain), humanized (a human antibody + a mouse CDR) and fully human [31]. MA-K2 whose properties were studied in this work is 65% humanized. In the next stage of our research we are planning to humanize it further and to conduct a series of in vivo experiments in primates using the humanized antibody.

CONCLUSIONS
We have characterized 4 chimeric monoclonal anti-TRBV9 antibodies (MA-K1, MA-K2, MA-K3, and MA-K4) different in the amino acid sequences of their hypervariable domain (CDR3). According to the literature, the TRBV9 family is associated with AS. The MA-K2 antibody was selected for further research based on its biochemical properties (Kd, Kdis, and specificity). It exhibited high specificity and cytotoxicity against the target. We are planning to further humanize the antibody and carry out a series of in vivo experiments in primates. If we succeed in eliminating the pathogenic T cell population carrying the TRBV9 gene segment, this monoclonal antibody will have the potential to become a drug candidate for AS therapy and a convenient tool for studying this disease.

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