Mining the human autoantibody repertoire: Isolation of potent IL17A-neutralizing monoclonal antibodies from a patient with thymoma

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Abbreviations: mAb, monoclonal antibody; IL17A, Interleukin 17A; HFF-1, Human Foreskin Fibroblasts; scFvs, single chain variable fragments; PBMCs, peripheral blood mononuclear cells; RT-PCR, Reverse transcription polymerase chain reaction; CMC, Chronic mucocutaneous candidiasis; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; FACS, fluorescence-activated cell sorting; huFc-γ1, human Fc-gamma 1; CDR, complementary-determining region.

Anti-cytokine autoantibodies have been widely reported to be present in human plasma, both in healthy subjects and in patients with underlying autoimmune conditions, such as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) or thymic epithelial neoplasms. While often asymptomatic, they can cause or facilitate a wide range of diseases including opportunistic infections. The potential therapeutic value of specific neutralizing anti-cytokine autoantibodies has not been thoroughly investigated. Here we used mammalian cell display to isolate IL17A-specific antibodies from a thymoma patient with proven high-titer autoantibodies against the same. We identified 3 distinct clonotypes that efficiently neutralized IL17A in a cell-based in vitro assay. Their potencies were comparable to those of known neutralizing antibodies, including 2, AIN457 (secukinumab) and ixekizumab that are currently in clinical development for the treatment of various inflammatory disorders. These data clearly demonstrate that the human autoantibody repertoire can be mined for antibodies with high therapeutic potential for clinical development.

Introduction

Monoclonal antibodies (mAbs) have become the treatment option of choice for an increasing number of important human diseases, including cancer and inflammatory disorders. While the very first therapeutic mAb approved in 1986 was entirely murine in sequence,1,2 newly developed and approved therapeutic mAbs are now commonly fully human or, at the very least, humanized.3 Numerous technologies are available for the selection or identification of human mAbs, such as: (1) phage display screening of large naive or semi-synthetic human libraries, typically followed by affinity maturation;4-6 (2) immunization of transgenic or transchromosomal (a.k.a. humanized) mice carrying human Ig loci and consecutive hybridoma screening;7-10 and (3) the cloning of “fully natural” human mAbs directly from human-derived peripheral B cells or plasma cells.11-15

To date, 13 human mAbs have been approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA), or are currently undergoing the approval process (for an regularly updated list of therapeutic mAbs see: http://www.landesbioscience.com/journals/mabs/about/ #background). Of these, 4 were selected by phage display and 9 were generated in humanized mice. Given the longstanding interest and intense research, it is only a matter of time until the first “fully natural” human-derived mAb will be approved for human use.

The most obvious targets for human-derived antibody repertoires are antigens associated with infectious agents, such as viruses and bacteria. To this end, the natural antibody repertoire could serve as a source for anti-infective mAbs. Additionally, given its critical role for host defense against many pathogens, the humoral immune system can be expected to be a rich source...
for high-affinity, anti-infective mAbs. Accordingly, human mAbs against numerous important infectious agents have been described, including influenza A, human immunodeficiency virus-1 and cytomegalovirus. Many of these antibodies have very favorable properties, including high potency and broad cross-protection, indicating that human-derived antibody repertoires do indeed exhibit a significant previously untapped therapeutic potential.

In contrast to infectious agents, many high-value targets of therapeutic interest, such as those associated with cancer or inflammatory disorders, are typically self-antigens. Since self-reactive antibodies could be detrimental to the host, the immune system has sophisticated tolerance mechanisms in place to prevent production of autoreactive antibodies. Nevertheless, appearance of autoantibodies has been described for various types of malignancies, including breast, ovarian, lung and prostate cancer. Their possible function in immune surveillance of cancer and potential use as biomarkers has been discussed extensively. Further, it has been shown that patient-derived anti-cancer autoantibodies can be cloned and produced recombinantly and may also have therapeutic potential.

Autoimmune repertoires may also be of interest for the targeting of certain inflammatory disorders. There is a plethora of literature describing the existence of anti-cytokine autoantibodies in healthy individuals and certain patients, including autoantibodies against interferon (IFN-α), IFN-γ, interleukin (IL)10, IL6, IL17A, IL22 and granulocyte-macrophage colony stimulating factor (GM-CSF). Surprisingly, anti-cytokine antibodies appear to be quite ubiquitous in healthy individuals, and there has been some speculation about their origin and specific function. In certain pathological conditions, elevated levels of autoantibodies may arise as a result of upregulated expression of certain cytokines and may influence disease progression. For instance, IL10-neutralizing autoantibodies have been reported in some patients with rheumatoid arthritis and appear to correlate with a milder form of the disease. Finally, patients with underlying autoimmunity, most notably those suffering from autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome or thymic malignancy, have been shown to produce various types of neutralizing anti-cytokine autoantibodies often causally linked to opportunistic infections. Chronic mucocutaneous candidiasis (CMC), for example, occurs in patients with APECED and is thought to be due to high-titer, neutralizing autoantibodies to the T-cell cytokines, IL17 and IL22, that regulate mucosal antibacterial and antifungal activity. Such neutralizing autoantibodies against these 2 interleukins were also identified in thymoma patients suffering from CMC, which implies that the immunodeficiency underlying CMC has an autoimmune basis. Taken together, these data suggest that the human autoantibody repertoire is a suitable source for the isolation of cytokine-neutralizing mAbs to treat inflammatory disorders.

We have previously described the anti-cytokine autoantibody profile of patients with thymic neoplasia. In this context, IL17A-neutralizing autoantibodies are of particular interest, given their therapeutic potential in psoriasis and various other inflammatory conditions. In this study, we identified a thymoma patient with high levels of IL17A-neutralizing autoantibodies. We report the successful isolation, cloning and characterization of several potent IL17A-neutralizing antibody clonotypes from this patient.

**Results**

Identification of a patient with IL17A-neutralizing autoantibodies

The patient described in this study is a 65 year-old female with a history of thymoma, CMC, and sinopulmonary infection with both nontuberculous mycobacteria and *Scedosporium apiospermum*. Her peripheral B cells (CD19+) numbered 112 cells/µliter (normal range 81–493). Patient plasma was screened for autoantibodies using a particle-based multiplex assay, yielding significant amounts of anti-IFN-α, anti-IL12, anti-IL17A, anti-IL17F, and anti-IL22 autoantibodies, none of which were identified in healthy controls in this study (N = 10, black boxes indicate min to max values with line at mean) (Fig. 1A), or a previous one. To evaluate the biological activity of anti-IL17A autoantibodies, IL17A-induced production of IL6 was analyzed in HFF-1 cells in the presence of healthy adult control (N = 5) or patient-derived plasma. Culture supernatants from cells incubated in the presence of control plasma demonstrated a 4.7-fold increase of IL17A-induced IL6, while those incubated with patient-derived plasma showed no increase in IL6 production (Fig. 1B).

Isolation of IL17A-specific human mAbs

Human mAbs were isolated using the eMAB® platform, a Sindbis virus-mediated mammalian cell display technology. First, IL17A-specific B cells were isolated from peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS). The immunoglobulin variable regions of the heavy and light chains were then amplified by RT-PCR, assembled to single-chain antibody (scFv) coding regions, and cloned into a Sindbis vector, generating an IL17A-focused library comprising γ heavy chain and κ and λ light chain variable regions. Sindbis virus was then generated from the library and used to infect baby hamster kidney (BHK) cells. Cells were stained for cell surface expression of IL17A-specific scFv using fluorescently labeled mouse antibody (scFv) coding region by RT-PCR. Sequencing of the PCR products revealed that 100% of the isolated heavy chain variable regions were IgG-derived (Table 1).
There was a very strong prevalence of one particular clonotype, arbitrarily named clonotype 1 (IGHV1–2), which was found in 85% of the clones (34 of 40 sequences). Significantly, this particular heavy chain was found paired with 9 different light chains of both κ and λ subtype. Finding the same heavy chain paired with several different light chains is not per se surprising given the combinatorial approach taken during library construction. The remaining 6 sequences consisted of 4 additional heavy and light chain pairs, 3 of which were unique.

Validation of isolated patient-derived human autoantibodies based on binding to and neutralization of IL17A as Fc-γ1 fusion proteins

In order to validate the isolated antibodies in terms of their specificity for IL17A, 20 representative scFvs comprising all selected heavy and light chain clonotype combinations were cloned and expressed in HEK-293T cells as human Fc-γ1 fusion proteins. The supernatants from transiently transfected cells containing scFv-huFc-γ1 antibodies were subjected to analysis by

![Figure 1. Detection of anti-IL17A autoantibodies and evaluation of their biological activity. (A) Particle-based multiplex determination of anticytokine autoantibodies for patient plasma (○) versus control plasma (boxes indicate healthy control values min to max with line at mean, N = 10). (B) HFF-1 cells were incubated with control (N = 5) or patient plasma and left unstimulated or stimulated with IL17A overnight. Culture supernatants were collected and evaluated for IL6 production. Shown is the fold-increase as the ratio of IL6 levels measured in stimulated vs. unstimulated culture supernatants. Average values represent data from 3 independent experiments.](image)

| Table 1. Summary of IL17A-specific antibodies identified |

| HC clonotype | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 3 | 4 | 5 |
|-------------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LC clonotype | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Clone name  | SM001 | SM019 | SM034 | SM007 | SM003 | SM020 | SM015 | SM055 | SM035 | SM010 | SM002 | SM027 | SM057 |
| ELISA binding (µg/ml) | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| Half-max binding (ng/ml) | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Neutralization | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− |

Antibody clones are grouped according to specific heavy chain (HC) and light chain (LC) clonotype combinations. HC and LC clonotypes were arbitrarily numbered 1–5 and 1–12, respectively. Clones in bold were chosen for molecular cloning as scFv-huFc-γ1 antibodies, small scale transient expression and initial ELISA screening (row “ELISA binding”). All ELISA binders were re-expressed at intermediate scale in serum free medium and quantified by Fc sandwich ELISA (row “Expression”). Half-maximal binding to immobilized IL17A was determined and the presence of neutralizing activity measured (bottom rows). Clones in bold and italic showed no (−) or only very weak (+/−) neutralizing activity and were not investigated further. Clones in bold and underlined showed neutralizing activities comparable to the benchmark antibodies.
ELISA to quantify scFv-huFc-γ1 expression levels, as well as evaluate IL17A binding. With the exception of clone 5M027, all clones bound their target antigen (Table 1). The remaining 19 scFv-huFc-γ1 antibodies were subjected to intermediate scale expression in serum-free medium for further analyses. The expression levels were sufficiently high (in the range of 5–23 μg/ml) for direct use in further ELISA and in vitro IL17A neutralization assays (Table 1). Half-maximal binding to immobilized IL17A was in the range of 0.8–3.8 ng/ml (Table 1), corresponding to an estimated affinity (K_D) of approximately 8–36 pM. Although skewed by avidity effects, these numbers indicated high affinity binding.

To further validate and select antibodies for cloning as human IgG1, the 19 scFv-huFc-γ1 antibodies were analyzed in an in vitro IL17A neutralization assay as unpurified proteins in cell culture supernatants on the human foreskin fibroblast cell line HFF-1. HFF-1 cells express the human IL17A receptor and are known to respond to IL17A stimulation by upregulating IL6 secretion.62,65 First, a dose response assay was performed by stimulating HFF-1 cells with serial dilutions of IL17A and quantifying the concentration of IL6 in culture supernatants by sandwich ELISA. A measurable IL6 response was observed at an IL17A concentration as low as 1 ng/ml, whereas saturation was reached at approximately 1 μg/ml (data not shown). Thus, to measure neutralization by antibodies, an IL17A concentration of 50 ng/ml in the linear range of the dose response curve was chosen. Increased IL6 expression was only observed in HFF-1 cells in the presence of IL17A, whereas in the absence of the cytokine (HFF-1 medium only) no IL6 expression could be detected. The same system was used to verify the biological activity of the in-house produced recombinant human IL17A, which was superior to commercially-available human IL17A in the assay (data not shown). Out of the 19 patient-derived anti-IL17A autoantibodies 11 clones represented 3 different heavy chain clonotypes, clonotype 1, clonotype 2 and 3 (Table 1), showed neutralizing activity, although 6 of them, 5M001, 5M031, 5M035, 5M040, 5M051 and 5M055, showed only very weak IL17A neutralization activity. The other 5 antibodies, 5M002, 5M007, 5M010, 5M012 and 5M024, showed neutralizing activities comparable to the reference MAb AIN457,66 a human anti-IL17A antibody developed by Novartis, and MAB317, a mouse monoclonal anti-IL17 antibody67 from R&D Systems. Both MAB317 and AIN457 efficiently neutralized IL17A activity with IC50s of 2.6 nM and 1.1 nM, respectively (Fig. 2). One clonotype of the patient-derived anti-IL17A autoantibodies represented by clones 5M007 and 5M024, neutralized IL17A with an IC50 of 30 pM and 63 pM, respectively. A second clonotype, represented by clones 5M010 and 5M012, had IC50s of 51 pM and 24 pM, respectively. Finally, antibody 5M002 had an IC50 of 0.94 nM (Fig. 2).

**Conversion of IL17A-specific scFv-Fc fusion proteins to human IgG1**

Based on the promising data obtained with scFv-huFc-γ1 antibodies binding to IL17A and the observation of the scFv-huFc-γ1 containing culture supernatants showing potency to neutralize human IL17A, we wanted to evaluate whether these properties can also be observed for the antibodies in the IgG1 format. The 5 most promising clones, 5M002, 5M007, 5M010, 5M012 and 5M024, were therefore selected for cloning and expression as human IgG1. Sequence alignments of the anti-IL17A antibodies are shown together with the 2 benchmark antibodies that were used throughout further characterization studies, AIN457 and an IgG1 version of the humanized mAb ixekizumab (LY2439821; Eli Lilly) (Fig. 3). Assignment of heavy and light chain genes was done using the IMGT Database.69 Antibodies 5M007 and 5M024, as mentioned before, are derived from the same heavy chain clonotype, which comprises the heavy chain gene segment IGH1–2 and which was found in 85% of the clones identified (clonotype 1). The 2 clones also share the same light chain clonotype comprising the lambda light chain gene segment IGL1–47. We selected both for further analyses because there are 2 point mutations in the light chain. One is in the hypervariable region complementary-determining region (CDR)1, where an arginine of 5M007 is exchanged by a serine in case of 5M024 (Arg31Ser), which might have an effect on the potency. Furthermore, there is one additional amino acid sequence variation in the J region, presumably a result of sequence ambiguity in the reverse primer used for amplification of the lambda variable regions (Fig. 3). Clonotype 2 is represented by 5M010 and 5M012, comprising the IGKV2D-28 kappa light chain and IGHV4–30 heavy chain V gene segments. The 2 clones show 2 amino acid mutations, a serine in CDR1 of the heavy chain of 5M010 is exchanged by an asparagine in 5M012 (Ser31Asn) and an additional amino acid variation is present in the J region of the light chain gene (Fig. 3), presumably a result of sequence ambiguity in the reverse primer used for

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**Figure 2.** Neutralization of IL17A-induced IL6 expression by scFv-huFc-γ1 expressed in HEK-293T culture supernatants. HFF-1 cells were treated with 50 ng/ml human IL17A in the presence or absence of serial dilutions of the indicated antibodies expressed as huFc-γ1 fusion proteins in HEK-293T culture supernatants. After 24 hours, supernatants were analyzed for IL17A-induced IL6 expression by IL6 sandwich ELISA, and the 50% inhibitory concentration (IC50) was determined. Benchmark antibodies are represented as dark bars. Error bars show 95% confidence intervals.
amplification of the lambda variable regions. As preliminary data showed no difference between 5M010 and 5M012, all shown results were restricted to 5M012. Finally, the third clonotype is represented by 5M002, comprising the IGKV1–39 kappa light chain and the IGHV4–59 heavy chain V gene segments. Additionally, the variable heavy and light chain gene sequences of AIN457, as well as from ixekizumab, are shown in the alignment. Both are currently being tested in Phase 3 clinical trials for treatment of psoriasis and other indications. The 2 benchmark antibodies do not share any significant sequence similarity in the CDRs with the sequences of the antibodies identified in this study.

### Binding of human monoclonal autoantibodies to human IL17A

In order to verify antigen specific binding of the expressed IgGs, the 4 anti-IL17A antibodies were analyzed for binding to human IL17A by ELISA in comparison to the benchmark antibodies AIN457 and ixekizumab. The EC50 values obtained from the ELISA are shown in Table 2 and concentration-dependent binding of anti-IL17A antibodies to human IL17A is shown in Figure 4A. AIN457 was used as a benchmark antibody and showed an EC50 value for binding to IL17A of 16.20 ± 3.23 pM. Except for clone 5M024, which had an EC50 value of 35.66 ± 25.94 pM, all other tested human anti-IL17A antibodies reached EC50 values in the range of the benchmark AIN457 (5M002: 21.23 ± 5.70 pM, 5M007: 18.28 ± 2.48 pM and 5M012: 19.97 ± 2.32 pM). The IgG1 version of ixekizumab (IgG4) was tested in a single experiment and also showed an EC50 value (12.8 pM) in the same range. The unspecific control mAb showed no binding to IL17A.

In addition to the determination of EC50 values via ELISA, the binding affinity of the identified IL17A autoantibodies was determined using Bio-Layer Interferometry (ForteBio Octet system). The results of this analysis are shown in Table 2. Due to the complexity of the experiment, it was only utilized as a tool to compare the 4 selected IgGs with the benchmark antibody AIN457. The antibodies were captured on anti-hlgG-Fc-Capture (AHC) Biosensors (ForteBio) and association of recombinant human IL17A was analyzed. For dissociation rates below the detection limit of the instrument (K\textsubscript{dis} = 10\textsuperscript{-5} 1/s), the K\textsubscript{D} value was calculated assuming a K\textsubscript{dis} (1/s) equals 10\textsuperscript{-5} divided by the measured K\textsubscript{on} (1/Ms). This implies that the real K\textsubscript{D} value is better, or in the worst case equal, to the calculated K\textsubscript{D}. Thus, it can be assumed that the affinities reported here are an underestimation of the real affinities. The reference antibody AIN457 showed a mean K\textsubscript{D} value of 133 ± 51pM. Similar K\textsubscript{D} values have been observed for the 4 clones 5M002, 5M007, 5M010, 5M012, and 5M024.

### Table 2. Comparison of IL17A autoantibodies based on binding to IL17A by ELISA (EC50), by Bio-Layer Interferometry (K\textsubscript{D}) and functional activity by in vitro IL17A neutralization (IC50)

| IgG   | EC50 (pM) | KD (pM) | IC50 (nM) | IC50 (nM) Exp. 4 |
|-------|-----------|---------|-----------|------------------|
| Ixekizumab | 12.8*     | 158.0   | 0.19 ± 0.019* |           |
| AIN457 | 16.20 ± 3.23 | 166 ± 23 | 0.43 ± 0.18 | 0.63 ± 0.001 |
| 5M002 | 21.23 ± 5.70 | 140.0   | 4.90 ± 3.11 | 9.29 ± 0.386 |
| 5M007 | 18.28 ± 2.48 | 269.0   | 0.16 ± 0.03 | 0.20 ± 0.003 |
| 5M012 | 19.97 ± 2.32 | 94.2    | 0.34 ± 0.11 | 0.42 ± 0.002 |
| 5M024 | 35.66 ± 25.94 | 237.0   | 0.15 ± 0.15 | 0.21 ± 0.012 |

Mean EC50 values (pM) were determined by IL17A binding ELISA in 3 independent experiments via analyses of concentration dependent binding of IgG1 (3000 pM to 4.12 pM) to 2 μg/ml human IL17A. K\textsubscript{D} values (pM) were determined using Bio-Layer Interferometry (ForteBio Octet) by capture of 10 μg/ml IgG1 using Anti-hlgG-Fc Capture (AHC) biosensors and association of 40 nM, 20 nM and 10 nM of recombinant human IL17A. In case of AIN457, ixekizumab, 5M002 and 5M012 the sensitivity of the dissociation constant k\textsubscript{dis} (1/s) was below the detection limit (<10\textsuperscript{-5}). For these IgGs, K\textsubscript{D} values were calculated using k\textsubscript{dis} (1/s) at 10\textsuperscript{-5} divided by kon (1/Ms). Mean IC50 values (nM) were determined by in vitro IL17A neutralization based on stimulation of IL6 expression by HFF-1 cells with 25 ng/ml IL17A in presence of serial dilutions of IL17A autoantibodies (10 μg/ml to 5.6 x 10\textsuperscript{-5} μg/ml). Mean IC50 values were derived from 4 independent experiments performed in duplicates. IC50 values from one representative experiment are shown for comparison reasons. Values derived from one experiment only are marked by an asterisk.
described for the scFv-huFc-monoclonal anti-IL17A autoantibodies with the closely related cytokine IL17F. The specificity of binding was analyzed using a particle-based multiplex assay (Fig. 4B). Each of the patient-derived antibody clones was characterized by an exquisite specificity, with no detectable crossreactivity with any of the cytokines tested. This is in striking contrast to mAb AIN457 which, under the experimental conditions chosen, displayed significant crossreactivity in striking contrast to mAb AIN457 which, under the experimental conditions chosen, displayed significant crossreactivity.

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Neutralization of IL17A using patient-derived human monoclonal anti-IL17A autoantibodies

The biological activity of anti-IL17A IgG1 was evaluated as described for the scFv-huFc-γ1 antibodies by measuring their potential to neutralize IL17A on HFF-1 cells stimulated by the referred cytokine. Upon stimulation of HFF-1 cells with 25 ng/ml IL17A in the presence of serial dilutions of anti-IL17 autoantibodies ranging from 10 μg/ml to 5.6 × 10⁻⁵ μg/ml, a dose dependent neutralization of IL17A, and hence decrease in IL17A-dependent IL6 expression of HFF-1 cells, was observed (Fig. 5). Two control antibodies with known IL17A-neutralizing activity were included in the experiment as benchmark antibodies, the IL17A-neutralizing mAb AIN457 and an IgG1 version of ixekizumab (IgG4).

With the exception of ixekizumab, all antibodies were assessed for neutralization 4 times in duplicates. The mean IC50 values of all 4 independent experiments are shown in Table 2. Both benchmark antibodies, AIN457 and ixekizumab IgG1, efficiently neutralized IL17A activity with IC50 values of 0.43 ± 0.18 nM and 0.19 nM ± 0.019 nM, respectively. Patient-derived anti-IL17A autoantibodies compared quite favorably to the reference mAbs in neutralizing IL17A. The 2 antibodies derived from the same heavy chain, clones 5M007 and 5M024, both neutralized IL17A with an IC50 of 0.16 ± 0.03 nM and 0.15 ± 0.15 nM, respectively, which is comparable to, or even exceeding, the neutralizing activity observed with the 2 benchmark antibodies AIN457 and ixekizumab. A similar IC50 was observed for 5M012 with 0.34 ± 0.11 nM, which is also in the range of the benchmark antibodies. Lower potency was observed for the 5M002 antibody, with an IC50 of 4.9 ± 3.11 nM. The comparison of their performances is based on the 4th experiment where ixekizumab was included (Fig. 5A) and IC50 values from experiment 4, which is representative for all independent experiments, are shown in Figure 5B.

In summary, by applying our fully human mAb discovery platform, eMAB®, we were able to isolate human anti-IL17A antibodies from a thymoma patient with high titer autoantibodies. Based on the characterization of a large set of unpurified human Fc-γ1 fusion proteins in HEK-293T cell culture supernatants in terms of binding and IL17A neutralization in vitro, we identified 4 promising clones representing 3 clonotypes for expression as human IgG1. The 4 patient-derived IL17A autoantibodies, 5M002, 5M007, 5M012 and 5M024, all showed binding to recombinant human IL17A in ELISA. The KD values obtained by Bio-Layer Interferometry indicated an affinity in the pM range comparable to values measured for AIN457 and ixekizumab. More importantly, when analyzing their potential to neutralize IL17A in an in vitro IL17A neutralization assay, all identified antibodies exhibited potent neutralizing activity. All of the tested human mAbs except for 5M002 showed IL17A neutralization activity in the range of the 2 benchmark antibodies, AIN457 and ixekizumab, which are currently in Phase 3 clinical trials.

Discussion

In the present study we demonstrate, for the first time, the isolation of fully human mAbs from a thymoma patient against an autologous target, IL17A. IL17A is an important T cell-derived cytokine involved in host defense against bacteria and fungi.

![Figure 4](image-url)
Abnormal regulation or presence of this cytokine in skin and joints, however, may lead to serious inflammatory and autoimmune disorders, including psoriasis, rheumatoid arthritis and psoriatic arthritis. Clinical data suggest that IL17A is a key "driver" cytokine in psoriasis that activates pathogenic inflammation; emerging cytokine-targeted mAb therapies therefore promise new treatment possibilities. The most advanced IL17A blocking agents are AIN457, a human mAb developed by Novartis and ixekizumab, a humanized IgG4 mAb, developed by Eli Lilly. Both agents are AIN457, a human mAb developed by Novartis and ixekizumab, a humanized IgG4 mAb, developed by Eli Lilly. Both antibodies, AIN457 and an IgG1 version of ixekizumab, were used in this study as benchmark antibodies because they are currently undergoing evaluation in Phase 3 clinical trials.

In contrast to the mAb AIN457, which was generated by immunization of transgenic mice engineered to express the human IgGκ repertoire, the patient-derived antibodies identified in this study were generated by the human immune system. Since these antibodies originated in the human body, they have undergone somatic hypermutation and affinity maturation and thus are characterized by high affinity. The study presented here represents the first assessment of the repertoire of IL17A-specific antibodies in a patient with neutralizing activity. In addition to the natural repertoire, the eMAB® technology allowed chain shuffling of light and heavy chains, thereby increasing the natural diversity of the obtained library. Interestingly, we found one predominant heavy chain (clonotype 1) that was paired with 9 different light chains, all of which were capable of binding IL17A. This indicates that antigen recognition is primarily mediated by the heavy chain. However, only one combination, represented by 5M007 and 5M024, was found to possess neutralizing activity in our in vitro IL17A neutralization assay. It is tempting to speculate that this particular pairing corresponds to the natural, patient-derived pairing and was therefore selected by maturation in the human body. The binding and neutralization data illustrated in this study support this hypothesis.

Binding to IL17A and potent neutralization of IL17A in vitro was observed for all 4 antibodies, 5M002, 5M007, 5M012 and 5M024, whether they were expressed as scFv-Fc-γ1 fusion proteins, or as fully human IgG1. However, we did observe significant, antibody format-dependent differences in the neutralization potencies of the 4 clones. Generally, the IgG1 format was less potent than the scFv-huFc-γ1, with differences ranging from 2.3-fold in the case of 5M024 to 14-fold in the case of 5M012. Different potencies of different antibody formats may simply be a reflection of differences in the bio-physical properties of the molecules. However, since we compared transiently expressed, unpurified scFv-γ1 with protein A-purified, acid-eluted IgG1 preparations, it is possible that, at least in the case of clone 5M012, the reduced potency of the IgG1 format may be due to an inherent pH-dependent lability of the antigen binding site. The identified IgG1 antibodies, 5M002, 5M007, 5M012 and 5M024, showed affinities to IL17A in the pM range, similar to the benchmark antibodies AIN457 and ixekizumab. Supporting the affinity data reported here, the mean KD obtained for AIN457 produced in-house (166 nM ± 23 nM) was in accordance with that reported in the Novartis patent application (US008119131B2), where the dissociation equilibrium constant for binding to IL17A was determined using BLAcore and resulted in a KD of 122 nM ± 22 nM.

The potencies of the patient-derived antibodies were evaluated and compared to the benchmark antibodies in an in vitro IL17A neutralization assay. Except for one of the patient-derived anti-IL17A antibodies, 5M002, which appeared to be ~10-fold less potent than AIN457, all other antibodies had neutralization activity in a similar range to AIN457. The potency of AIN457 was determined in our IL17A neutralization assays to be about 5-fold higher (IC50 of 0.43 nM ± 0.18 nM at 1.4 nM IL17A) than that reported previously (IC50 of 2.1 nM ± 0.1 nM at 1.87 nM IL17A). The observed differences may be due to the IL17A-responsive cell line used or other differences in the assay set-up. Significantly, the 2 antibodies 5M007 and 5M024 showed slightly better neutralizing activity than AIN457. In agreement with the binding data, the 2 single amino acid

![Figure 5. Neutralization of IL17A by fully human monoclonal anti-IL17A autoantibodies. (A) IL6 response measured at OD405 upon stimulation of HFF-1 cells with 25 ng/ml IL17A in presence of serial dilutions of identified anti-IL17A autoantibodies and benchmark antibodies AIN457 and ixekizumab (10 nM to 5.6 x 10^-5 µg/ml). (B) Diagram of IC50 values (nM) determined by detection of IL6 secreted from HFF-1 cells upon stimulation with IL17A (25 ng/ml) in presence of serial dilutions (10 nM to 5.6 x 10^-5 µg/ml) of 5M002, 5M007, 5M012, and 5M024 (light bars) in comparison to the 2 benchmark antibodies AIN457 and ixekizumab (dark bars). IC50 values were calculated in GraphPad Prism using nonlinear regression (log inhibitor versus response) for analyses. Error bars show 95% confidence intervals.](Image)
differences in the variable light chain did not affect potency. The one amino acid difference in framework 4, encoded by the J gene segment, is likely to be a result of primer ambiguity during library construction. In addition, it is conceivable that the amino acid exchange in the CDR1 is not a result of affinity maturation, but the result of PCR amplification during library preparation from IL17A specific B-cells.

The second benchmark antibody, ixekizumab (IgG1) was comparable to AIN457 and the patient-derived natural human antibodies in terms of binding and neutralization potency. We tested ixekizumab here only as the IgG1 version (ixekizumab is used as IgG4 in clinical studies) because the effector functions, which are mediated by the Fc part of IgG1 and IgG4, were not thought to be relevant for the neutralization or binding assays described here. Still, the activities of IgG1 and IgG4 antibodies may differ in vivo, since IgG1 is a significantly more potent mediator of effector functions than IgG4. It is important to mention that anti-cytokine autoantibodies have clear pathological roles in certain diseases, which underscores the need to understand how these autoantibodies can occur apparently under non-disease circumstances and how they can be used to prevent or treat disease. Multiple aspects of an autoantibody may influence disease activity, including IgG subtype, antibody titer and binding affinity. Treatment options for targeting IL17A have raised concerns about fungal infections, since people with inborn genetic deficiencies of IL17RA and IL17F suffer from CMC. However, no fungal infections have been reported following the use of ixekizumab (IgG1) because the effector functions, which are mediated by the Fc part of IgG1 and IgG4, were not thought to be relevant for the neutralization or binding assays described here. Still, the activities of IgG1 and IgG4 antibodies may differ in vivo, since IgG1 is a significantly more potent mediator of effector functions than IgG4. It is important to mention that anti-cytokine autoantibodies have clear pathological roles in certain diseases, which underscores the need to understand how these autoantibodies can occur apparently under non-disease circumstances and how they can be used to prevent or treat disease. Multiple aspects of an autoantibody may influence disease activity, including IgG subtype, antibody titer and binding affinity. Treatment options for targeting IL17A have raised concerns about fungal infections, since people with inborn genetic deficiencies of IL17RA and IL17F suffer from CMC. However, no fungal infections have been reported following the use of ixekizumab (IgG1) because the effector functions, which are mediated by the Fc part of IgG1 and IgG4, were not thought to be relevant for the neutralization or binding assays described here.

In summary, anti-IL17A autoantibodies can be readily isolated from selected patients and have the potential to efficiently neutralize IL17A. This approach warrants further development and holds promise for the treatment of inflammatory and other diseases.

**Materials and Methods**

**Preparation of plasma and peripheral blood mononuclear cells**

The patient was seen at the National Institutes of Health and consented to evaluation and treatment under institutional review board (IRB)-approved protocol 01-I-0202. Control (healthy adult) PBMCs and plasma were obtained through the National Institutes of Health Blood Bank under appropriate IRB-approved protocols. Plasma was obtained by centrifugation of whole blood at 2000g for 10 minutes; the remaining cellular component was processed for PBMCs by density gradient centrifugation as described. PBMCs were frozen at 5 × 10^6 cells/vial in freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide) and stored in liquid nitrogen until use.

**Particle-based multiplex assay**

Plasma from patients and healthy controls were screened for anti-cytokine autoantibodies using a particle-based technology described previously. Briefly, 21 sets of differentially fluorescing magnetic beads (Bio-Rad) were conjugated to 2.5 μg recombinant human GM-CSF (R&D Systems, 215-GM-010/CF), IFN-α (R&D Systems, 11101–2/CF), IFN-γ (R&D Systems, 285-IF-100/CF), IFN-λ1 (R&D Systems, 1598-IL-025/CF), IFN-λ2 (R&D Systems, 1587-IL-025/CF), IFN-λ3 (R&D Systems, 5259-IL-025/CF), IFN-α (Peprotech, 300–02)), IL1α (eBioscience, 34–8364), IL4 (eBioscience, 34–8049), IL6 (eBioscience, 34–8069), IL7 (eBioscience, 34–8079), IL10 (eBioscience, 34–8109), IL12 (R&D Systems, 219-IL-025/CF), IL15 (eBioscience, 34–8159), IL17A (R&D Systems, 317-ILB-050/CF), IL17F (eBioscience, 34–8479), IL18 (R&D Systems, B003–5), IL22 (R&D Systems, 782-IL-010/CF), M-CSF (eBioscience, 34–8789), TNFα (eBioscience, 34–8329), or TNFβ (R&D Systems, 211-TBB-010/CF), respectively. Beads were combined and incubated for 1 hour with subject or control plasma at 1:100 dilution, washed, and incubated with PE-labeled goat anti-human IgG (eBioscience, 12-4998-82) before being run in a multiplex assay on the Bio-plex (Bio-Rad) instrument. Fluorescence intensity for each bead type was plotted as a function of antibody titer (Graphpad Prism, version 5.0c). Specificity of recombinant, purified anti-IL17A antibodies was investigated in the same manner, at a concentration of 0.2 μg/ml.

**Detection of IL17A neutralizing activity in patient plasma**

For functional evaluation of anti-IL17A autoantibodies, HFF-1 cells (ATCC SCRC-1041) were used as described previously, and cultured in complete medium consisting of DMEM with 15% FCS, 2 mM glutamine, 20 mM Hapes, and 0.01 mg/mL penicillin/streptomycin in 5% CO₂. Culture supernatants were harvested and analyzed for IL17A-induced IL6 levels using a bead-based cytokine assay (Bio-Plex Streptavidin-PE, Bio-Rad, #171–304501) and processed according to the manufacturer’s specifications.

**Production of recombinant human IL17A**

The nucleotide sequence encoding the mature form of human IL17A (aa 20–155) was cloned into a pET-based bacterial expression vector to allow for expression of a His-tagged fusion protein. The IL17A expression vector was transformed into the E. coli strain BL21 Star™ (DE3) (Life Technologies, C6010-03) and expressed by induction of 1 mM isopropyl-b-D-thiogalactopyranoside for 4 hours at 25°C. Bacterial pellets were lysed in lysis buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM DTT, 500 mM NaCl, 0.1% Triton X-100) and insoluble inclusion bodies were first washed with wash buffer 1 (50 mM Tris-HCl pH 8, 1 mM EDTA, 10 mM DTT, 500 mM NaCl, 0.1% Triton X-100, 2 M urea), then with wash buffer 2 (50 mM Tris-HCl pH 8, 1 mM EDTA, 10 mM DTT, 500 mM NaCl, 0.1% Triton X-100, 3 M urea), and finally with wash buffer 3 (50 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM DTT, 150 mM NaCl). Inclusion bodies were then re-suspended in solubilization buffer (6 M Guanidine-HCl/0.1 M Tris/10 mM DTT pH 8.5) at a total protein concentration of 14 mg/ml.
Briefly, 10\(^7\) PBMCs were stained for the biological activity with that of commercially-available IL17A (R&D Systems, 317-ILB-050) (data not shown). Native folding of the protein was confirmed by comparing its dimer formation by reducing and non-reducing SDS-PAGE. Finally, the buffer of the purified IL17A was exchanged to 50 mM Na-phosphate, 300 mM NaCl, pH 6.5 by gel filtration on a Sephadex G25 column and the concentration increased by ultrafiltration. The resulting soluble IL17A was checked for purity and dimer formation by reducing and non-reducing SDS-PAGE.

**Antibody screening by mammalian cell display**

Human antibodies were isolated using methods described previously.\(^{11,16}\) Briefly, 10\(^7\) PBMCs were stained for the presence of anti-IL17A cell surface immunoglobulin and various cell surface markers, and 40 IgM/IgD/CD3/CD14-negative, CD19/anti-IL17A-positive cells presumably representing IL17A-specific memory B cells were sorted on a FACSArta\(^b\) III flow cytometer (Becton Dickinson). The immunoglobulin variable regions of the heavy and light chains were then amplified by RT-PCR and assembled to single-chain antibody (scFv) coding regions. One library, comprising 10\(^7\) heavy chain and 10\(^6\) light chain variable regions, was generated and cloned in the Sindbis cell surface display vector pCYT-DISP.\(^{78}\) The resulting library consisted of 2.5 \(\times\) 10\(^8\) independent transformants, a large excess over the theoretical diversity of 1.6 \(\times\) 10\(^8\) clones required to cover all possible combinations of heavy and light chains. Sindbis virus with a titer 2.1 \(\times\) 10\(^7\) pfu/ml was then generated from the library and used to infect BHK cells at a low multiplicity of infection (MOI), to ascertain expression of a single antibody species per infected cell. Single cells displaying IL17A-specific scFv antibodies were then sorted into individual wells of 24-well plates containing 50% confluent BHK feeder cells using fluorescently-labeled antigen. Upon virus spread (2 d post sorting), the infected cells were tested by FACs analysis for IL17A-binding to identify virus clones encoding IL17A-specific scFv antibodies. Supernatants from wells containing infected BHK cells displaying highly IL17A-reactive scFvs were harvested and the corresponding scFv coding regions amplified by RT-PCR and sequenced.

**Transient expression of scFv-Fc antibodies**

Fusion proteins were generated carrying an N-terminal human scFv fused to a C-terminal human Fc-\(\gamma\)1 domain. Thus, scFv coding regions were digested with the restriction endonuclease SfI and cloned into the expression vector pCEP-SP-SfI-huFc-\(\gamma\)1. This vector is a derivative of the episomal mammalian expression vector pCEP4 (Invitrogen, V044–50), carrying the Epstein-Barr virus replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) to permit extrachromosomal replication, and contains a puromycin selection marker in place of the original hygromycin B resistance gene. The resulting plasmids drive expression of scFv-huFc-\(\gamma\)1 fusion proteins under the control of a CMV promoter. Small scale expression of the scFv-huFc-\(\gamma\)1 fusion proteins was done by transiently transfecting the expression vectors into HEK-293T cells on 24 well plates using Lipofectamin Plus (Invitrogen, 15338-100). From each initial PCR product, 4 minipreps were separately transfected. Three days later, culture supernatants were harvested and subjected to ELISA to verify expression and antigen-specific binding. Clones with verified expression and IL17A-specific binding were sequence-verified and subjected to intermediate scale expression. For this, HEK-293T cells were transfected in 6 well plates using Lipofectamin Plus and, one day later, re-plated onto poly-lysine coated 10 cm plates. After 3 days, the medium was replaced by fresh, serum-free medium. Four days later, the serum-free culture supernatants were harvested, passed through 0.22 \(\mu\)m sterile filters, and stored at 4℃ until further use.

**Cloning, expression and purification of mAb AIN457 and ixekizumab**

The sequence of the human mAb AIN457 was taken from the patent application US008119131B2. Variable heavy and light chain sequence information for ixekizumab was used from the IMGT database.\(^{79}\) The heavy chain and light chain coding regions were generated by total gene synthesis (GeneArt AG, Regensburg, Germany) and combined into a single, EBNA-based expression vector, pCB15 essentially as already described.\(^{80}\) Expression of AIN457 and ixekizumab was done by transfecting the expression vector into HEK-293T cells, using Lipofectamin Plus (Invitrogen, 15338-100). For large scale production and purification, stable protein-expressing cells were enriched by selection in the presence of 1 \(\mu\)g/ml puromycin (Sigma, P8833–10MG). Pools of resistant cells were maintained in serum-free medium on Poly-D-Lysine (Sigma, 27964–99–4) coated dishes for 4 weeks. Supernatant containing AIN457 and ixekizumab was collected twice a week, filtered through a 0.22 \(\mu\)m sterile filter (Millipore, SCGVU11RE), and purified by protein A affinity chromatography. Quantification was carried out via analytical size exclusion chromatography.

**Cloning, expression and purification of 5M002, 5M007, 5M010, 5M012 and 5M024**

5M024 and 5M010 were cloned into pCB15 as described for AIN457 and ixekizumab. 5M002, 5M007 and 5M012 were cloned and expressed as IgG1s in pEX1, which is based on pCB15. The main difference is the replacement of the second CMV promoter by an IRES motif which is placed before the IgG heavy chain gene. Furthermore, pEX1 already contains sequences encoding light and heavy chain constant domains, which facilitate IgG1 assembly by enabling a one-step cloning procedure via recombination of light and heavy chain variable region coding sequences into the plasmid. 5M002, 5M007 and 5M012 were cloned into pEX1 via recombination using the In-Fusion® HD Cloning Plus Kit (Clontech, 639648).
Table 3. Variable heavy (VH) and variable light (Vk, Vl) chain specific primers used for cloning of 5M002, 5M007 and 5M012 as IgG1

| IgG          | Forward                                      | Reverse                        |
|--------------|----------------------------------------------|--------------------------------|
| 5M002-VH     | TCCGGTGCCCATCTCTGAAGCGTAGCCAGGAGTCCGAGAAGTCG | CTTGGTGAGGCGGTCGAGAGGGGTGTTCCCG |
| 5M002-Vk     | TTTCTCCACAGGTTCATGTGACATCCAGATGACCCAGTCCACCTCTTCG | GCGACAGCTTGG          |
| 5M007-VH     | TCCGGTGCCCATCTCTGAAGCGTAGCCAGGAGTCCGAGAAGTCG | CTTGGTGAGGCGGTCGAGAGGGGTGTTCCCG |
| 5M007-Vl     | TTTCTCCACAGGTTCATGTGACATCCAGATGACCCAGTCCACCTCTTCG | GCGACAGCTTGG          |
| 5M012-VH     | TCCGGTGCCCATCTCTGAAGCGTAGCCAGGAGTCCGAGAAGTCG | CTTGGTGAGGCGGTCGAGAGGGGTGTTCCCG |
| 5M012-Vk     | TTTCTCCACAGGTTCATGTGACATCCAGATGACCCAGTCCACCTCTTCG | GCGACAGCTTGG          |

according to the manufacturer’s specification. Variable light (kappa or lambda) and variable heavy chain coding regions were amplified from the respective scFv-Fc expression vectors pCEP-SP-Sf-huFc-γ1 with sequence specific primers (see Table 3) using standard PCR conditions (Phusion High Fidelity DNA Polymerase, Biozym, F-530S). The resulting fragments were flanked by sequences, allowing recombination into the linearized pEX1-kappa plasmid (in case of 5M002 and 5M012 containing a kappa light chain) and pEX1-lambda plasmid (in case of 5M007 which contained a lambda light chain). After verification of the correct heavy and light chain sequences, expression and purification were performed as described for AIN457 and ixekizumab.

ELISA analyses

Three types of analyses were carried out: 1) an Fc Sandwich ELISA to quantify scFv-huFc-γ1 expression levels; 2) an antigen-specific ELISA to verify IL17A-specific binding and to determine EC50 values of scFv-huFc-γ1 and IgG1; and 3) a sandwich ELISA to quantify IL17A induced IL6 expression levels in HFF-1 cell culture supernatants. Thus, to quantify scFv-huFc-γ1 expression levels and verify IL17A-specific binding, plates were coated overnight at 4°C with goat F(ab')2 anti-human Fcy antibody (Jackson ImmunoResearch Laboratories, 109-006-098) and recombinant human IL17A at a concentration of 2 μg/ml in coating buffer (0.1 M NaHCO3, pH 9.6), respectively. The plates were then washed with PBS/0.05% Tween®20 and blocked for 2 h at 37°C with 3% BSA in PBS/0.05% Tween®20. After washing for quantification of scFv-huFc-γ1 expression levels, plates were incubated for 2 hours at room temperature with 3-fold serial dilutions of culture supernatants in PBS/0.05% Tween®20 containing 1% BSA, starting with a 5-fold dilution. The previously purified Influenza A M2-specific scFv-huFc-γ1 D00516 was used as a standard in the Fc Sandwich ELISA to allow for quantification of expression levels. The plates previously coated with recombinant human IL17A were incubated for 1 hour at room temperature with 3-fold serial dilutions of IgG1 starting at 3 nM concentrations of IgG1 in PBS/0.05% Tween®20 containing 1% BSA. Specifically-bound antibodies were detected after extensive washing with HRPO-labeled, Fc-specific, goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, 109-035-098). After washing with PBS/0.05% Tween®20, plates of the Fc-Sandwich ELISA were developed with a 0.4 mg/ml solution of 1,2-ortho-phenylenediamine dihydrochloride in citric acid buffer (35 mM citric acid, 66 mM Na2HPO4, pH 5.0) containing 0.01% H2O2. After 15 min, the reaction was stopped with a 5% solution of H2SO4 in H2O, and plates read at 450 nm on an ELISA reader (Biorad Benchmark). The IL17A binding ELISA was developed using ABTS (Sigma, A1888). After 30 minutes the plates were read at 405 nm on an ELISA reader (Bio Tek Synergy 2). EC50 values for IgG1 binding to IL17A were determined in GraphPad Prism using nonlinear regression (curve fit) and log(inhibitor) vs. response parameter.

The IL6 Sandwich ELISA was carried out using BD OptEIA™ Human IL6 ELISA Set (BD Biosciences, 555220) according to the manufacturer’s specifications. For detection of HFF-1 cell derived IL6 by the anti-human IL6 capture antibody, culture supernatants were diluted 1:3 in PBS/0.05% Tween®20 containing 1% BSA. ELISA plates were developed using ABTS (Sigma). After 30 min the plates were read at 405 nm on an ELISA reader (Bio Tek Synergy 2).

IL17A neutralization assay with human scFv-huFc-γ1 and fully human IgG

IL17A-induced expression of IL6 in human fibroblasts and its inhibition by neutralizing anti-IL17A antibodies was measured in a neutralization assay. Thus, HFF-1 cells were cultivated in Dulbecco’s Modified Eagle Medium (Gibco, 31966-047) supplemented with 15% fetal calf serum (PAA) and 1% Antibiotic-Antimycotic (Gibco, 15240-062) and seeded in 96-well plates at a density of 10^5 cells per well. The next day, cells were either left unstimulated or stimulated with either 50 ng/ml or 25 ng/ml human IL17A when analyzing scFv-huFc-γ1 and fully human IgG, respectively. For neutralization, IL17A was preincubated for 30 minutes at room temperature with 3-fold serial dilutions of anti-IL17A antibodies starting at a concentration of 10 μg/ml which were applied onto the HFF-1 cells. After 24 hours at 37°C and 5% CO2, supernatants were harvested and induced IL6 expression was measured by Sandwich ELISA, as described above. The concentration of induced IL6 was determined by including a serial dilution of recombinant human IL6 (Peprotech, 200-06) as a standard starting at 100 ng/ml. IC50 values were determined in GraphPad Prism using nonlinear regression (curve fit) and log(inhibitor) vs. response (variable slope) analyses.
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