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ORIGINAL RESEARCH

Preliminary Investigation and Therapeutic Efficacy Determination of a Novel Anti-IL-17A Antibody, Indikizumab

Ashok Kumar Patra[1], Shreenath Nayak[1], Anandita Moharana[1], Purusottam Ojha[1], Sanjeet Kumar Das[1], Jabed Akhtar[1], Bishwaranjan Giri[1], Sujay Singh[1,2]

[1]Protein Expression Lab, Imgenex India Pvt. Ltd. E-5, Bhubaneswar, Odisha, India; [2]Sujan Biologics, Inc., San Diego, California, USA

Correspondence: Ashok Kumar Patra, Director, Biologics Development, Imgenex India Pvt. Ltd, E 5, Infocity, Technology Corridor, Chandaka Industrial Area, Bhubaneswar, Odisha, India, 751024, Email apatra@imgenexindia.com

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Purpose: The study aimed to develop and characterize Indikizumab, a novel humanized anti-IL-17A monoclonal antibody (mAb), for potential therapeutic use in inflammatory indications such as psoriasis, psoriatic arthritis, rheumatoid arthritis, and ankylosing spondylitis.

Methods: The research involved the purification of IL-17 isoforms, epitope mapping, affinity ranking, and comparative binding assessment of anti-IL-17 antibodies. The study also included cell-based neutralization assays and in vivo studies using mouse models to evaluate the efficacy of Indikizumab.

Results: Indikizumab demonstrated a high binding affinity (K_D=27.2 pM) and specificity for IL-17A, with comparable potency to Secukinumab. In cell-based neutralization assays, Indikizumab effectively neutralized the effects of IL-17A and demonstrated a statistically significant reduction in plasma KC (Keratinocyte) levels in a mouse model. In imiquimod-induced psoriasis mouse model, Indikizumab showed potential in reducing the psoriasis index.

Conclusion: Indikizumab represents a promising therapeutic option for inflammatory indications with its high binding affinity, specificity for IL-17A, and effectiveness in neutralizing IL-17A effects in vivo.

Keywords: monoclonal antibody, neutralization assay, psoriasis, inflammation. keratinocyte

Introduction

"Psoriasis" is a chronic immune-mediated inflammatory disease that causes thick, itchy patches on the skin and is characterized by unchecked keratinocyte proliferation. This disorder is prevalent worldwide, and affects approximately 2%–3% of the population in Western countries and[1,2] approximately 1.2% of the Indian population.[3] This disorder though not associated with mortality, but significantly associated with comorbidity including cardiovascular disease, obesity, dyslipidemia, and many other metabolic syndromes.[4] It is also reported to be associated with psychiatric disorders like depression, anxiety, and suicidal tendencies.[5,6] Psoriasis is associated with the upregulation of several cytokines and has a complex pathogenesis; a fusion of immunologic, environmental, and genetic factor leading to upregulation and activation of immune pathways thought to be key driver of initiation of psoriasis.[7] In psoriasis and related inflammatory indications cytokine IL17 is thought to be a key regulator of disease progression. This cytokine is extensively expressed in T helper 17 (Th17) cells which are a novel fraction of T lymphocytes,[8,9] In several research studies the role of IL-17–Th17 cell pathway in many inflammatory and autoimmune diseases and role in host defense has been well established. In yet another study role of cytokine interleukin-23 as a key mediator of T helper 17 activation that leads to IL-17 production has been established[10,11] According to a theory, inflammatory dendritic cells that have invaded the skin site release IL-23 and IL-12, which encourage Th1 cells, Th22 cells, and T cells to produce cytokines including IL-17, IFN-γ, TNFα, and IL-22, which stimulate keratinocytes and intensify psoriatic inflammation.[12–15] Considering the

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role of IL23 in the production of IL17, it is postulated that IL23/IL17 pathway has a critical role in the pathogenesis of psoriasis and is recognized as the dominant pathway for disease development. Apart from the role of cytokines such as IL12, IL17 & IL23 in psoriasis development, genetical analysis of healthy subjects and patients indicated the presence of several psoriasis risk loci suggesting a significant genetic contribution towards the development of this disorder.[16,17] Furthermore, overwhelming evidence points to the critical function that IL-17 plays in the host's defense against *S. aureus* and *Candida albicans* infections of the cutaneous and mucosal tissues and[18–21] for upholding the intestinal epithelial barrier.[22,23] In addition to the above, IL-17 also plays a critical role in the production of neutrophils activator IL6 that has been linked to pro-inflammatory as well as anti-inflammatory activity.[24] Out of the several IL17 isoforms, IL-17A is the subtype that has been studied the most in terms of both physiological and pathological circumstances, including psoriasis.[25] This cytokine has been produced by various cell types such as Tc17, Th17 cells, tissue-resident memory T (T_{RM}) cells, invariant natural killer T cells (iNKT), innate lymphoid cell (ILC)-3, mucosal-associated invariant T (MAIT) cells and gamma delta T cells.[5] The release of cytokines and chemokines is stimulated by IL-17A, in turn, these cytokines and chemokines activate neutrophils and memory T-cells, bring them to the site of injury or inflammation, and maintain a proinflammatory state.[26] IL-17 was initially identified as cytotoxic T lymphocyte antigen 8 (CTLA 8), associated with an open reading frame (ORF) in the T-cell tropic Herpesvirus saimiri virus.[27] This gene was cloned from an activated T cell and is now formally known as IL-17A. Subsequently, other members of this cytokine family such as IL-17B to IL-17F, and their receptors, IL-17RA to IL-17RE were also discovered. Structurally, IL-17A can coexist with IL-17F as an A/F heterodimer or as a homodimer (A/A).[28] Pathogenic T-cells that produce IL-17A are involved in the pathogenesis of autoimmune disorders, such as ankylosing spondylitis, rheumatoid arthritis, psoriatic arthritis, and psoriasis. Up to 24% of psoriasis patients also have psoriasis arthritis.[6] Due to the complex nature of these anti-inflammatory diseases' initiation and progression, controlling disease and improving the quality of life of the patient is a challenge. Therefore, apart from small molecule therapy, the current therapeutic strategy for these inflammatory diseases also involves the intervention of biologics such as monoclonal antibodies.[29]

Currently, several anti-IL17A biological agents are approved for the management of psoriatic arthritis, psoriasis, and ankylosing spondylitis. Some of them are; Secukinumab, a completely human monoclonal antibody that targets IL-17A (Brand name: Cosentyx marketed by Novartis), Ixekizumab, humanized IgG4 specific for IL-17A/F (Brand name: Taltz, marketed by Eli Lily) and Bimekizumab, humanized IgG1 antibody that suppresses IL-17A and IL-17F at the same time (Brand name Bimzelx, developed by UCB). In this study, we described the generation of Indikizumab a humanized IgG1 variant IL-17A-neutralizing antibody and partial characterization suggesting it's binding affinity and specificity. Indikizumab binds to human IL-17A with high affinity but does not bind to other IL-17 family members. Indikizumab effectively inhibits the interaction between IL-17A and its receptor in binding assays and potently blocks IL-17A-induced GROα or Keratinocyte secretion in cell-based assays. In an in vivo pharmacokinetic mouse model, indikizumab efficacy in blocking mouse KC secretion has been established. These informational resources offer an extensive research assessment of Indikizumab for which the efficacy, potency, and safety will be demonstrated in mice models.

Materials and Methods

Interleukin 17 isoform genes were synthesized by GeneScript Inc. *E. coli* strains such as BL21DE3, DH5α were purchased from Novagen. The nucleotide sequence information of Secukinumab, Ixekizumab, and Guselkumab were derived from the amino acid sequence information listed in the Drugbank database and were synthesized by GeneScript Inc. Buffer components such as Tris, Phosphate buffers, NaCl, electrophoresis reagents, IPTG (Isopropyl β-D-1-thiogalactopyranoside), Glycerol etc., were purchased from MP Biomedicals. NexGen HM Protease Inhibitor (cat. No. BPBOI1) from Biopioneer Pvt. Ltd. Mouse CXCL1-ELISA MAX™ Deluxe Set, (Cat No-447504) and Human CXCL1/GRO alpha ELISA kit (ABTS), (LS-F57479) were purchased from BioLegend and LS Bio respectively. C57BL/6 and BalbC mice were brought from Imgenex India's animal house facility. All other chemicals used were according to their parentheses.

IL-17 Expression and Purification

The gene sequences of IL-17A, B, C, D & F isoforms were obtained from UniProt database and the literatures. The nucleotide sequences were chemically synthesized and cloned into pET28a+ vector between NcoI and XhoI sites (GenScript, Inc) so that the sequence would have C-terminal 6xHis amino acids for IMAC purification. All the plasmids were transformed to *E. coli* BL21DE3 cells and expressed using 1 mM IPTG induction following the standard expression process and further purified using IMAC resins (Roche, Cat No. 05893801001). Details of the sequences used are listed in the Table 1.

Hybridoma Development

Every animal study was carried out in compliance with and authorized by the research standards of Imgenex India Pvt. Ltd., Animal House facility and Animal Ethics Committee, India. A carrier-free recombinant human IL-17A protein was used to immunize mice as per the standard process. Mice were immunized subcutaneously with 20 µg of recombinant IL-17A at every alternative day for 21 days. Mice were bled on 22nd day and an indirect ELISA (Enzyme Linked Immunosorbent Assay) was performed to determine the titre of antibodies developed against immunogen. The mouse with high titre of antibody was given an intraperitoneal pre-fusion booster on 23rd day. The antibody secreting B-cells were isolated from the lymph nodes after sacrificing the mouse and fused with F_0 myeloma cells on 25th day. The fused cells were cultured in HAT (hypoxanthine-aminopterin-thymidine) selection media for 4–5 days followed by HT (Hypoxanthine-Thymidine) supplement medium for 14 days. Then supernatants of the hybridomas were tested by indirect ELISA to check the production of anti-hIL-17A antibodies. Initially, 21 anti-IL-17A antibodies secreting hybridomas were selected, and further selection was done based on ELISA absorbance. The eight best hybridoma clones were selected and then expanded for further testing. The antibodies produced by these clones were tested for specificity by Western blotting against the human IL-17A protein, and 4 best hybridomas clones were selected for further expansion and were sub-cloned for generation of monoclonal antibody.

Subclone Selection and Characterization

The subclones were selected by ELISA by testing them on serially diluted recombinant human IL-17A Protein. Four subclones depending upon their ability to detect human IL-17A protein in sub-nanogram level were finally selected. Further, the selected subclones were also tested for their specificity by carrying out an ELISA using some unrelated proteins. Out of the four clones, ABM2A18 was selected due to its higher affinity and specificity towards IL-17A.

Antibody Sequencing and Humanization by CDR Grafting

The antibody that showed promise in the functional assay was used for humanization using CDR (Complementarity determining region) grafting method. Antibody sequencing was carried out as per standard procedure. In brief, the total RNA was extracted from hybridoma cells, followed by RT-PCR. The antibody genes were sub-cloned into a mammalian expression vector. A number of single colonies were picked and sequenced. Using the mouse antibody as a reference, five heavy chain and five light chain human acceptors were chosen. The CDRs of the mouse antibody were directly grafted

Table 1 Amino Acid Ranges of IL-17 Family and Their Calculated Molecular Weight

Interleukins	UniProt I.D.	Amino Acid Range	No. of AA	NMW	NMW with HIs Tag
IL-17 A	Q16552	24–155	132	15.12	16.32
IL-17 B	Q9UHF5	21–180	160	18.15	19.42
IL-17 C	Q9P0M4	19–197	179	19.73	21.00
IL-17 D	Q8TAD2	16–202	187	20.39	21.59
IL-17 F	Q96PD4	31–163	133	14.90	16.15

Abbreviations: NMW, Nominal Molecular Weight as per calculation; AA, Amino acids.

into the human acceptor frameworks to get the sequences of the five humanized heavy chains and light chains. Using NCBI Ig-Blast, the mouse antibody's variable domain sequences were searched against the database of human germline (http://www.ncbi.nlm.nih.gov/projects/igblast/). For each heavy chain and light chain, five different human acceptors (ie variable domains of human with high similarity to the mouse antibody) were selected. The humanized variable domain sequences were produced by substituting the human CDRs for the mouse acceptor CDRs. VH1, VH2, VH3, VH4, and VH5 were the humanized heavy chains, and VL1, VL2, VL3, VL4, and VL5 were the humanized light chains. Twenty-five humanized antibodies (5 heavy chains × 5 light chains) were produced and subjected to affinity ranking. The affinities of these antibodies were measured using Biacore T200.

Humanized IgG Production

Codons of mouse-human chimeric and humanized heavy and light chains were optimized for HEK293 cells. The optimized sequences were inserted into expression vector followed by Maxiprep of transfection grade plasmid. Transient expression in 100 mL HEK293 (ATCC-CRL-1573) cells were carried out as per standard procedure. The chimeric and humanized antibodies were purified by protein-A affinity chromatography. The production yields of chimeric and humanized antibodies are shown in Table 2.

Specificity

Wells of 96-well ELISA plates were coated with IL-17 family member recombinant proteins at 0.5 µg/mL (50 ng/well) in duplicate and incubated for 16 hours at 4°C. Different concentrations of Indikizumab at 0 µg/mL, 0.016 µg/mL, 0.08 µg/mL, 0.4 µg/mL, 2 µg/mL, or 10 µg/mL were then added to the wells of ELISA plate. After incubation of two hours at room temperature, the plates were washed four times using the washing buffer. Subsequently, HRP-conjugated anti-human Ig secondary antibody was added to the plates. The ELISA plate reader was used to measure the colorimetric signal. The production of the color signal was directly correlated with the level of indikizumab binding to the test proteins. The assay was repeated twice.

Pharmacodynamic Assay in Mice

The C57BL/6 mice were administered intravenously with Indikizumab (n=3/group, 8–12-week-old) one hour before administering a human IL-17A subcutaneous (SC) injection. Blood samples were taken two hours after the dose of IL-17A, and the level of CXCL1 chemokine in the plasma, was determined using ELISA. As an isotype control antibody, humanized anti-Her2 antibody was used. Using the two-way analysis of variance, the CXCL1 chemokine levels were compared pairwise between treatments. Concentration of chemokine CXCL1 secretion values were interpreted from the equation "$y = -7E\text{-}06x^2 + 0.0094x + 0.0419$", and the values were plotted to describe the effect of the drug treatment.

Table 2 Production Yield of Chimeric and Humanized Variants (VH+VL) Yield (Mg/100 Ml)

	VL1	VL2	VL3	VL4	VL5
VH1	0.09	0.31	0.3	Not Expressed	1.55
VH2	0.02	0.16	0.04	Not Expressed	0.75
VH3	0.1	1.07	0.58	Not Expressed	1.38
VH4	Not Expressed	Not Expressed	0.05	Not Expressed	0.45
VH5	0.01	0.33	0.42	Not Expressed	1.31

Note: Mouse-Human Chimeric Antibody=0.04.

In vivo Functional Assay

BALB/c mice were bred at Imgenex India. A daily topical dose of 62.5 mg of commercially available IMQ cream (5%) (IMIQUAD, from Glenmark) was applied to 8 to 11 wk of age of mice on the shaved back of the right ear for 5 or 6 consecutive days, translating in a daily dose of 3.125 mg of the active compound. It was found empirically that in mice, this dosage produced the most appropriate and consistent level of skin inflammation (data not shown). Similar treatment was given to control mice using a Vaseline control cream.

Results

Purification of IL-17 Isoforms

All five isoforms were cloned into pET28a vector and designed to have C-terminal 6xHis tag for affinity purification. IMAC was used to purify the isoforms to homogeneity. As the proteins were expressed as inclusion bodies, purification of proteins from inclusion bodies were adopted.[30] On-column refolding of the proteins was carried out by continuous gradient passing (8M urea to 2M urea) buffer (50 mM Tris, 2M Urea, pH 8, 300 mM NaCl, 0.4 M L-Arginine, 20 mM reduced glutathione, 2 mM oxidized glutathione) for 24 hours at 16°C. Protein was eluted from the column with increasing concentration of Imidazole. After elution, as per SDS-PAGE result, the fractions were pooled for concentration and buffer exchanged to 50mM Tris, pH-8.0, 300mM NaCl, 0.4M L-Arginine) through Amicon membrane concentrator, concentrated sample was run on a gel to check purity. As could be seen in the SDS-PAGE image, all the proteins have very prominent single bands of respective molecular weight indicating that the proteins were purified to >90% purity. At this moment IL-17 isoforms were pure enough for binding and specificity studies (Figure 1).

Epitope Identification for IL-17A Antibody

To recognize the interrelationship between human IL-17A and Indikizumab, studies were conducted to ascertain which epitope this antibody is targeting. A peptide array with 40 overlapping 15-mer peptides from mature IL-17A protein (amino acids 24–155) was created by PepperPrint, Germany, and was assayed according to PepperPrint protocol. Based on this study, Indikizumab binds to KNFPRTVMVNLNIHN peptide representing amino acids 39–53 in IL-17A protein. Blocking experiment was done to confirm this mapping (Data not shown).

Indikizumab Development and Production

Codons of mouse-human chimeric and humanized heavy and light chains were optimized for expression in HEK293 cells. The optimized sequences were inserted into expression vectorfollowed by Maxiprep of transfection grade plasmid

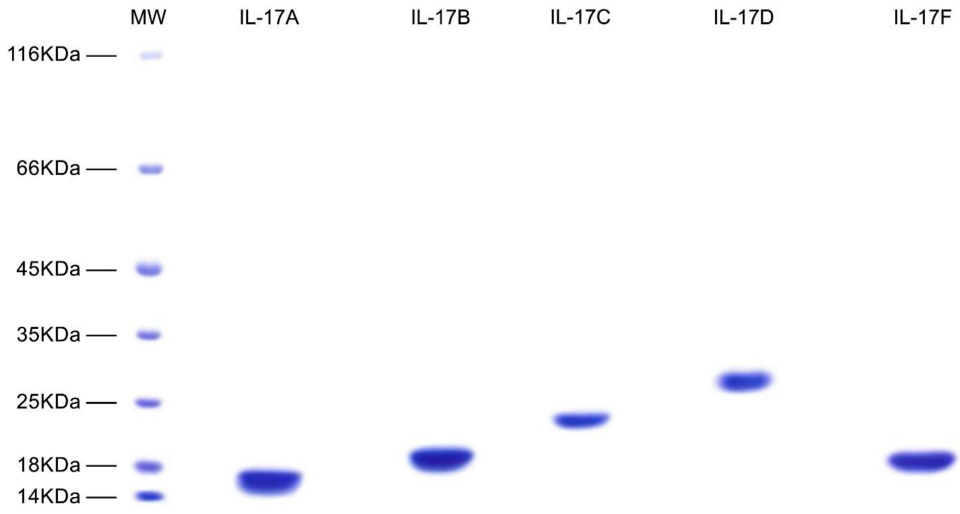

Figure 1 Purity analysis of IL17 isoforms by Electrophoresis (4–20% gradient SDS-PAGE). 10µL of each purified IL-17 isoform was loaded on to the 4–20% gradient SDS-PAGE. As shown in the PAGE analysis, all the five proteins are reasonably pure enough to carry out ELISA and other assays.

preparation. Transient expression in 100 mL HEK293 cells were carried out as per standard procedure. Purification of the humanized and chimeric antibodies was accomplished by Protein-A affinity chromatography. The production yields of chimeric and humanized antibodies are shown in Table 2. Out of 25 different constructs 7 constructs did not express. From the remaining 18 construct only five constructs showed expression 10 mg or more per liter of culture in the shake flask. The VL4 in combination with any of the VHs did not result in expression of the antibody, whereas, VH4 showed some expression only with VL3 and VL5. On the other hand, VL5 in combination with all the VHs resulted in high expression of antibdoy. These 18 constructs were taken to study affinity ranking using OctateRed96e.

Affinity Ranking by Kinetic Assay

Eighteen humanized variants that expressed were ranked based on their off-rates using OctetRed 96e. In 1×Kinetics assay buffer (1×PBS, 0.05% Tween-20, pH 7.4), the assay was run at 30°C. At 1000 rpm, the samples were stirred. AHC sensors were humidified for 15 minutes before analysis. The chimeric IgG and humanized variants were captured by goat anti-human Fc-γ immobilized on the AHC (Anti-Human IgG Capture) sensor. The association was allowed for 120 seconds. A single concentration (100 nM) of analyte (ie antigen protein IL17A) was used. The dissociation was monitored for the next 300 seconds. To compare the off rates of chimeric and humanized variants, the experimental raw data were normalized at the beginning of the dissociation (120 seconds) (Figure 2). Table 3 shows the percentage of remaining analyte after 300 seconds of dissociation. The higher the percentage of antigen remained on the sensor, the slower the off rate. Among the 18 combinations of Heavy and Light chains VH4+VL3 and VH4+VL5 combinations did not show any interaction indicating that the VH4 sequence was not a favorable sequence for whole antibody formation as well as not good for the antigen: antibody interaction. Though VL3 in combination with VH1, VH2 & VH3 showed better affinity ranking in Kinetic assay; however, their expression level is much less in comparison to VL5 in combination with the same Heavy chains. After 300 seconds of dissociation, combinations that hold ~90% of the analytes based on the Langmuir model of 1:1 interaction[31] were considered

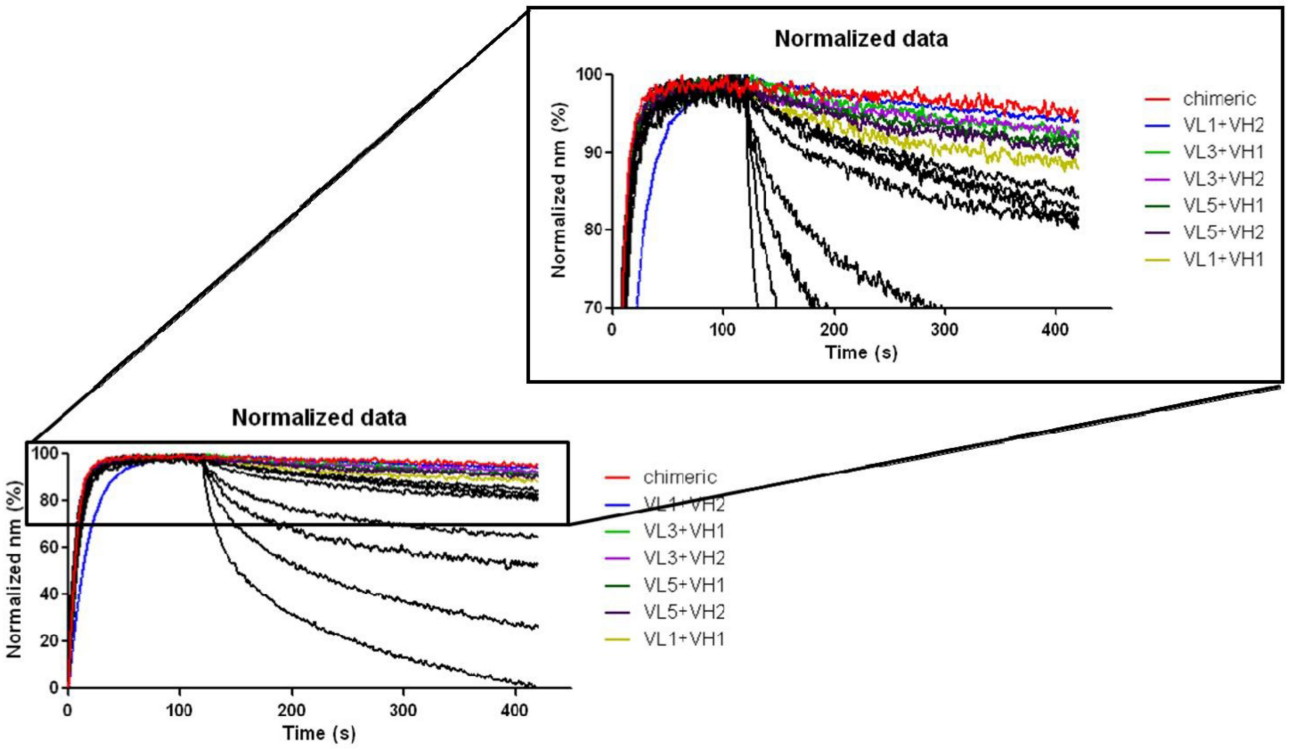

Figure 2 Binding behavior analysis and affinity ranking of humanized antibodies on AHC sensor using OctetRed96. Humanized antibody variants were ranked based on their off-rates using OctetRed 96. The assay was performed at 30°C in 1×Kinetics assay buffer (1×PBS, 0.05% Tween-20, pH 7.4). Agitated samples were captured by humidified AHC sensors. The association was allowed for 120 seconds. One concentration (100 nM) of analyte (ie antigen protein) was used. The dissociation was monitored for 300 seconds. In order to compare the off rates of chimeric and humanized variants, the experimental raw data were normalized at the beginning of the dissociation.

Table 3 Percentage of Antigen Remained on Sensor at 420s

	VL1	VL2	VL3	VL4	VL5
VH1	88.09	80.61	92.77	N.A.	90.73
VH2	94	81.78	91.93	N.A.	90.08
VH3	64.63	53.18	83.09	N.A.	84.31
VH4	N.A.	N.A.	No Interaction	N.A.	No Interaction
VH5	0.21	2.35	7.12	N.A.	26.27

Note: Control: Mouse-Human chimeric antibody shows 95.36%.
Abbreviation: N.A, Not Available.

for further analysis. This model proposes that one ligand binds to one analyte making a complex that follows pseudo-first order kinetics and it is assumed that binding is equivalent and independent of all binding sites. Based on the expression level as well as affinity ranking result, three best combinations were selected for further analysis of affinity by BiaCoreT200. They were VH1+VL5, VH2+VL5 & VH3+VL5.

Affinity Measurement

Affinity measurement using Biacore T200 was carried out following GE's handbook. A cycle of binding analysis consists of 3 steps: (i) capture of the ligand (ie humanized IgG) onto the sensor chip surface by the immobilized anti-Human antibody; (ii) binding of the analyte (ie antigen protein, increasing concentration ranging from 0.5 nM to 8 nM) to the ligand; (iii) separation of the attached analyte. Sensor surface was regenerated after each binding cycle using the manufacturer's recommended conditions. At 25°C, each binding cycle was conducted. The binding affinities of variants VH1+VL5, VH2+VL5, and VH3+VL5 are shown in Figure 3 and Table 4. As can be seen in the experimental graph the

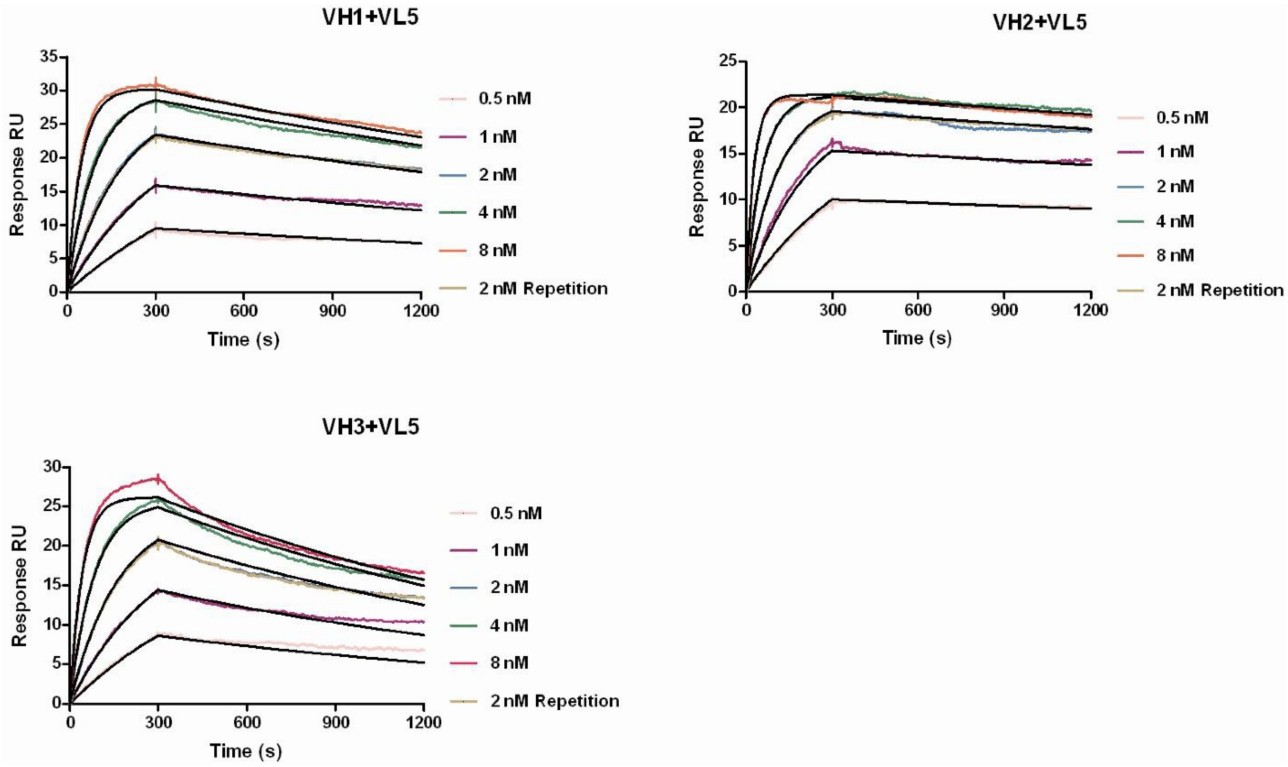

Figure 3 Antibody-antigen affinity measurement using Biacore T200. Affinity measurement using Biacore T200 was carried out following GE's handbook. A binding analysis cycle consists of three steps: (i) capture of the ligand (ie humanized IgG) onto the sensor chip surface by the immobilized anti-Human antibody; (ii) binding of the analyte (ie antigen protein, increasing concentration ranging from 0.5 nM to 8 nM) to the ligand; (iii) dissociation of bound analyte. Sensor surface was regenerated after each binding cycle using the manufacturer's recommended conditions. All binding cycles were run at 25°C.The binding affinities of variants VH1+VL5, VH2+VL5 and VH3+VL5 are shown. Black curves represent the fit of Langmuir 1:1 interaction model to experimental data. The antigen concentrations are 0.5 nM, 1nM, 2nM, 4nM and 8 nM; experiment at antigen concentration of 2nM was repeated for reference.

Table 4 Binding Affinity of Selected Humanized Variants

IgG	k_a(1/Ms)	k_d (1/s)	K_D (M)	R_{max} (RU)	Chi² (RU²)	U-value
VH1+VL5	2.60E+06	2.99E-04	1.15E-10	30.67	0.167	1
VH2+VL5	4.30E+06	1.17E-04	2.72E-11	21.42	0.117	3
VH3+VL5	2.94E+06	5.72E-04	1.95E-10	26.82	0.474	1

ligand and analyte association occurred within the first 200 seconds followed by a brief window of equilibrium constant of 90–100 seconds. The dissociation of the ligands and analytes was monitored till 1200 seconds. It is observed that the rate of association (k_a) was higher, rate of dissociation (k_d) is slower, and the dissociation constant (K_D) was lower in case of VH2+VL5 antibody, suggesting that VH2+VL5 combination was the best among all three heavy and light chain combinations. Considering the above results, VH2+VL5 combination of anti-IL17A antibody was taken for production and assessment. This antibody was named as "**Indikizumab**".

Comparative Binding Assessment of Anti IL-17 Antibodies

Both heavy and light chain IgG cDNAs with kappa chain leader sequence were chemically synthesized and cloned into IMGX-GS vector system. For comparative analysis, genes coding for heavy and light chains of Secukinumab (anti IL17A, Cosentyx; Novartis), Guselkumab; (anti IL-23/p19, Tremfya; Johnson & Johnson) and Ixekizumab, (anti-IL-17A/F, Taltz; Eli Lily) were also synthesized, transiently expressed in Expi-CHO cells and purified (Figure 4). Though Guselkumab is not an anti-IL-17A antibody, this was synthesized to provide some information on comparative specificity of the Indikizumab. Except Guselkumab all other antibodies are showing some degree of binding affinity to IL-17A antigen (Figure 5) in immune affinity assessment assay, whereas, all the antibodies show no binding affinity in specificity assessment assay (Figure 6) towards IL-17 B, C, and D. Very little or no binding affinity was observed against IL-17F. Our In-house antibody Indikizumab as determined from the above ELISA shows similar and selective binding to IL-17A in comparison to Secukinumab. Hence, to determine the effective binding saturation concentration @ 50% (EC_{50}), different concentration (10 ng-5120 ng) of IL-17A was coated in the ELISA plate and after following the SOP

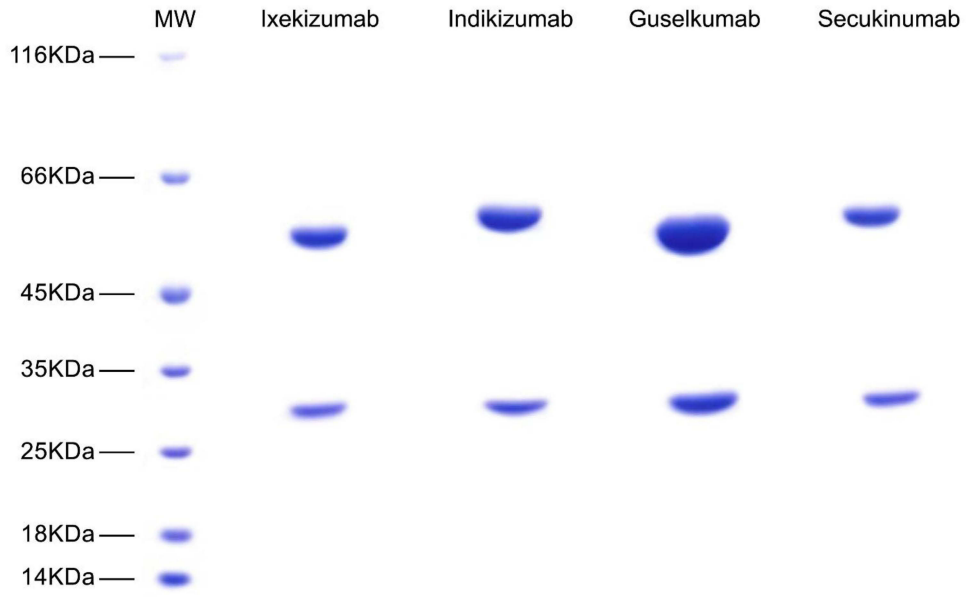

Figure 4 Purity analysis of anti-IL17 antibodies produced in transiently transfected CHO-K1 cell line by Electrophoresis (4–20% gradient SDS-PAGE). 10 µL of affinity (MabSure Select, Cytiva) purified antibodies were treated in the SDS-PAGE sample buffer for reduction. Boiled samples were loaded on to the gel for electrophoresis. All the four antibodies are relatively pure and the light chain and heavy chains migrated at the desired molecular weight in SDS-PAGE.

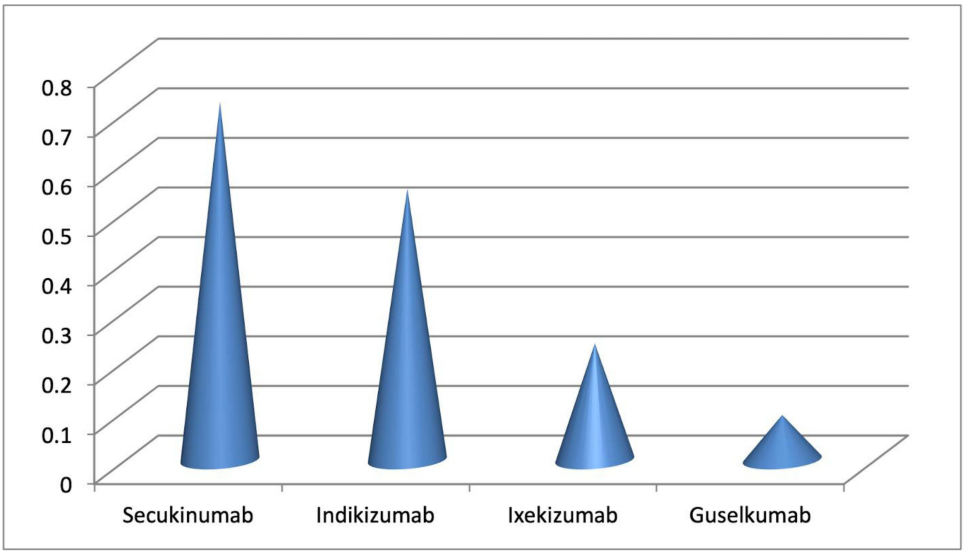

Figure 5 Comparative Immuno-affinity assessment of antigen-antibody by Enzyme Linked Immunosorbent Assay. 200 ng of E. coli expressed recombinant IL-17A was coated in the plate and above listed goat anti-human IgG-Fc-HRP was employed as the secondary antibody at a dilution of 1:5000, whilst antibodies (100 ng/well) were utilized as the primary antibody. Secukinumab and Ixekizumab are showing variable degree of affinity whereas, Guselkumab does not show any binding suggesting its specificity towards IL-23, at the same time our in-house developed Indikizumab shows binding affinity at par with Novartis Secukinumab. Indikizumab; humanized antibody against IL17A, Ixekizumab; humanized antibody against IL-17 A/F, Guselkumab; human monoclonal antibody that binds to the interleukin (IL)-23 p19 subunit specifically and prevents it from interacting with the IL-23 receptor; Secukinumab is a human IgG1κ monoclonal antibody neutralizes IL-17A.

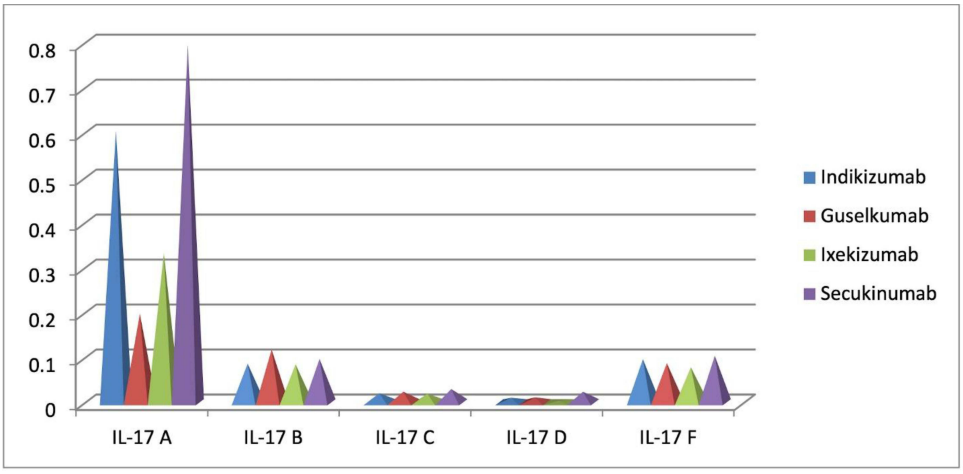

Figure 6 Determination of Binding specificity of anti-IL17 antibodies to IL17 isoforms. 200 ng of recombinant E. coli expressed IL-17A, B, C, D, F were coated in the plate and above listed Goat anti-Human IgG-Fc-HRP was employed as the secondary antibody at a dilution of 1:5000, primary antibodies used at 100 ng/well. Except Guselkumab all other antibodies are showing some degree of binding affinity to IL-17A antigen. All the antibodies show no binding affinity towards IL-17B, C, and D. Very little or no binding affinity is observed against IL-17F. Indikizumab; humanized antibody against IL17A, Ixekizumab; humanized antibody against IL-17A/F, Guselkumab; completely human monoclonal antibody that binds to the interleukin (IL)-23 p19 subunit specifically and prevents it from interacting with the IL-23 receptor; Secukinumab is a human IgG1κ monoclonal antibody neutralizes IL-17A.

absorbance was measured at 450 nm (Figure 7). The effective concentration determined as 371.8 ± 1.967 and 362.2 ± 2.127 ng for Indikizumab and Secukinumab, respectively.

Cell-Based Neutralization Assay

Numerous cell types, such as keratinocytes, fibroblasts, mesothelial cells, epithelial cells, and leukocytes, express IL-17A receptors. It has been demonstrated that IL-17A can cause fibroblasts and other cells to release either mouse or human CXCL1, the keratinocyte chemoattractant, or growth-regulated oncogene (GRO) α. It has been shown that human IL-17A binds to mouse IL-17A receptor. NIH/3T3 fibroblast cells from mice were utilized to investigate the neutralization of

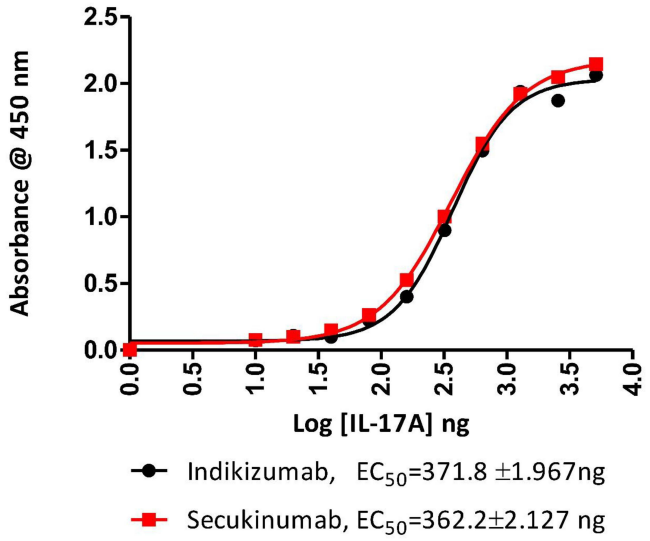

Figure 7 Comparative In vitro efficacy study between Indikizumab and Secukinumab by ELISA. Different concentration of recombinant- IL-17A (10ng-5120ng/well) were coated in the 96 well ELISA plate and incubated overnight at 4°C. Indikizumab and (Secukinumab) produced in CHO-K1 were used as the primary antibody at concentration of 100 ng/well. After the washing procedure binding signal was amplified and detected using Goat-Anti-Human IgG-Fc (1:5000 dilution). TMB Substrate used at 1x from 5x Stock. Statistical analysis was done using Log [Antigen] vs response- variable slope.

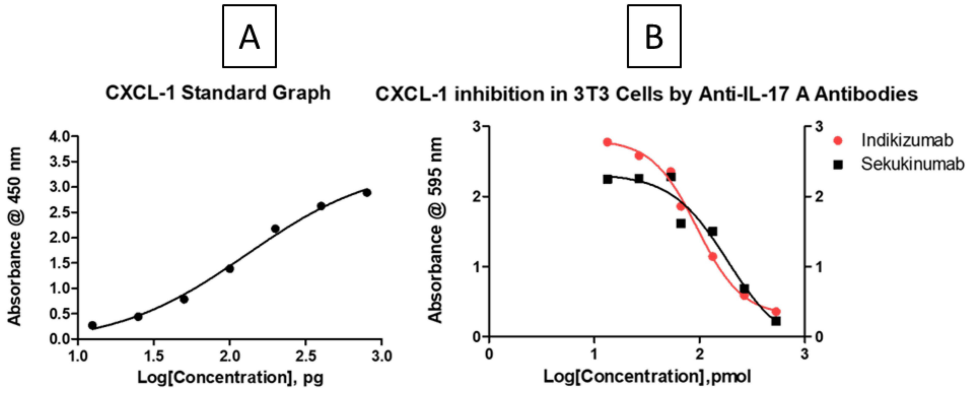

Figure 8 Comparative analysis of CXCL-1 inhibition in NIH 3T3 cells by Indikizumab and Secukinumab. (**A**). CXCL-1 Standard Graph; CXCL-1 (0–800 pg/well). (**B**). 3T3 cells were treated with pre-incubated mixture of Recombinant- IL-17A (60 ng/well) with Indikizumab & Secukinumab (13–533 pmol/well) for >60 hours. Culture supernatant was harvested and tested in ELISA. CXCL-1 capture antibody was coated in the 96 well ELISA plate and kept overnight. Following the SOP of blocking, binding and washing, detection antibody at concentration 100 ng/well was added. After the washing procedure binding signal was amplified and detected using Avidin-HRP (1:1000 dilution). TMB Substrate used at 1x from 5x Stock. Statistical analysis was done using Non-linear regression of Log (Concentration) vs response. At 95% Confidence Intervals; Log [IC_{50}] = Indikizumab =1.976± 0.058; Secukinumab = 2.26±0.28 pmol.

human IL-17A's effect on CXCL1 production (Figure 8). Efficacy of Indikizumab was compared with Secukinumab for neutralization of CXCL-1 and found to be at par.

Pharmacodynamic Study

To evaluate indikizumab neutralization, in vivo assay was developed, mice were injected with recombinant human IL-17A, and an assessment of cytokine level alterations was carried out. The injection of human IL-17A to C57BL/6 mice resulted in a dose- and time-dependent increase in mouse plasma KC levels. In a dose-dependent manner, indikizumab was able to reduce KC production induced by human IL-17A in the plasma of C57BL/6 mice. Compared to isotype control treatment, the mice treated with 20 µg of indikizumab showed a statistically significant decrease in plasma KC. These findings show that indikizumab was effective in in vivo neutralizing human IL-17A (Figure 9).

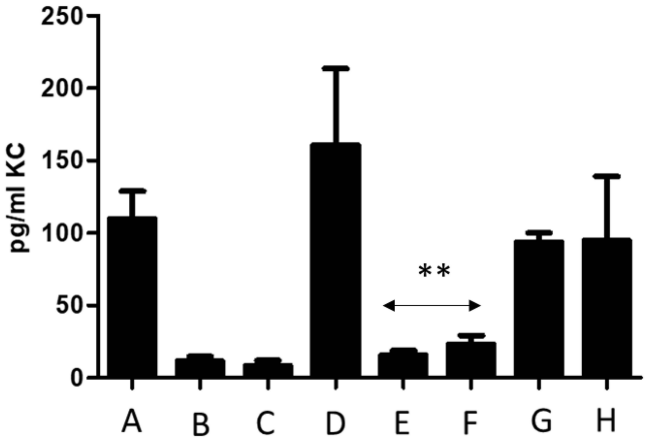

Figure 9 Indikizumab inhibits Human IL17A induced KC (Keratinocyte) secretion in C57BL/6 mice. C57BL/6 mice were given subcutaneous injections of human IL-17A (3 μg) one hour after receiving an IV injection of indikizumab (1 mg/kg, 0.1 mg/kg, 0.01 mg/kg, or 0.001 mg/kg) (ie, 20 μg, 2 μg, 0.2 μg, and 0.02 μg per mouse, respectively). Using ELISA, KC levels were determined 2 hours post human IL-17A injection. n=3 mice per group. Indikizumab treatment accounts for 68.99% of total variance. P value=0.0065** (If Indikizumab Treatment has no effect overall, there is a 0.65% chance of randomly observing an this big (or bigger) in an experiment of this size. The effect is considered very significant.). Group A-Human IL-17A 3 μg/mouse, B-Indikizumab 20 μg/mouse, C-Human IgG1 Isotype control 20 μg/mouse, D-Human IL-17A +Isotype control IgG1 20 μg/mouse, E- Human IL-17A+Indikizumab 20 μg/mouse, F- Human IL-17A+Indikizumab 2 μg/mouse, G- Human IL-17A+Indikizumab 0.2 μg/mouse, H- Human IL-17A+Indikizumab 0.02 μg/mouse.

In vivo Efficacy Study on Induced Plaque Psoriasis

In-house bred 8 to 12 weeks old BALB/c mice were taken in 4 groups (each group = 8 numbers of mice) received daily 62.5 mg of topical dose of commercially available IMQ cream (5%) (IMIQUAD, from Glenmark) on the shaved back for 6 consecutive days, translating in a daily dose of 3.125 mg of the active compound. It was found empirically that in mice, this dosage produced the most appropriate and consistent level of skin inflammation. After 6 days of drug treatment, antibody treatment was started. Group A, Group B, Group C, and Group D received buffer without any antibody, mouse monoclonal antibody, Indikizumab, and Secukinumab, respectively. Antibody treatment was at 48 μg/mouse/day single dose. Effect of drug IMIQUAD and effect of antibody were visually observed under three major categories in a scale of 1–4; Erythema (Redness), Induration (skin thickness), and Desquamation (scaliness) of the skin. Observed values were plotted against days of treatment (Figure 10).

Even though there was reversal of the plaque psoriasis in the control group after drug withdrawal, which could be attributed to incomplete or partial plaque psoriasis formation due to drug treatment; gradual decrease in the plaque psoriasis lesion in the treated group indicated that there is significant effect of Indikizumab in comparison to that of the Secukinumab.

Discussion

This report summarizes the development of Indikizumab, a novel humanized anti-IL-17A mAb. The selection of humanized Indikizumab included steps such as affinity ranking based on the binding affinity to antigen IL-17A,[32] cell based assay indicating efficacy and potency, and primary in vivo assay indicating efficacy. Analysis of various binding parameters indicated that a molecule with an extremely high affinity for antigen binding (K_D=27.2 pM) and low rate of dissociation has been identified, humanized, and sequence has been chemically synthesized and expressed in CHO cells. Statistical analysis of antibody-antigen binding studies suggested that VH2+VL5 complex has slow off rate, means longer half-life of the antibody-antigen complex. Binding affinity of Indikizumab and other related antibodies to IL-17A upon comparison showed variable degree of affinity, highest being shown by Secukinumab followed by Indikizumab and Ixekizumab. However, Guselkumab as expected showed negligible binding affinity. Indikizumab directly prevents IL-17A from binding to IL-17RA (data not shown) but showed negligible binding affinity towards other family members like IL-17B, IL-17C, IL-17D and IL-17F, suggesting its high specificity towards IL-17A. Indikizumab's closest competition is Secukinumab. Hence, efficacy of cell-based neutralization assay was compared with Secukinumab. Chemokines play important role in the recruitment and activation of T cells, macrophages, and neutrophils in psoriatic

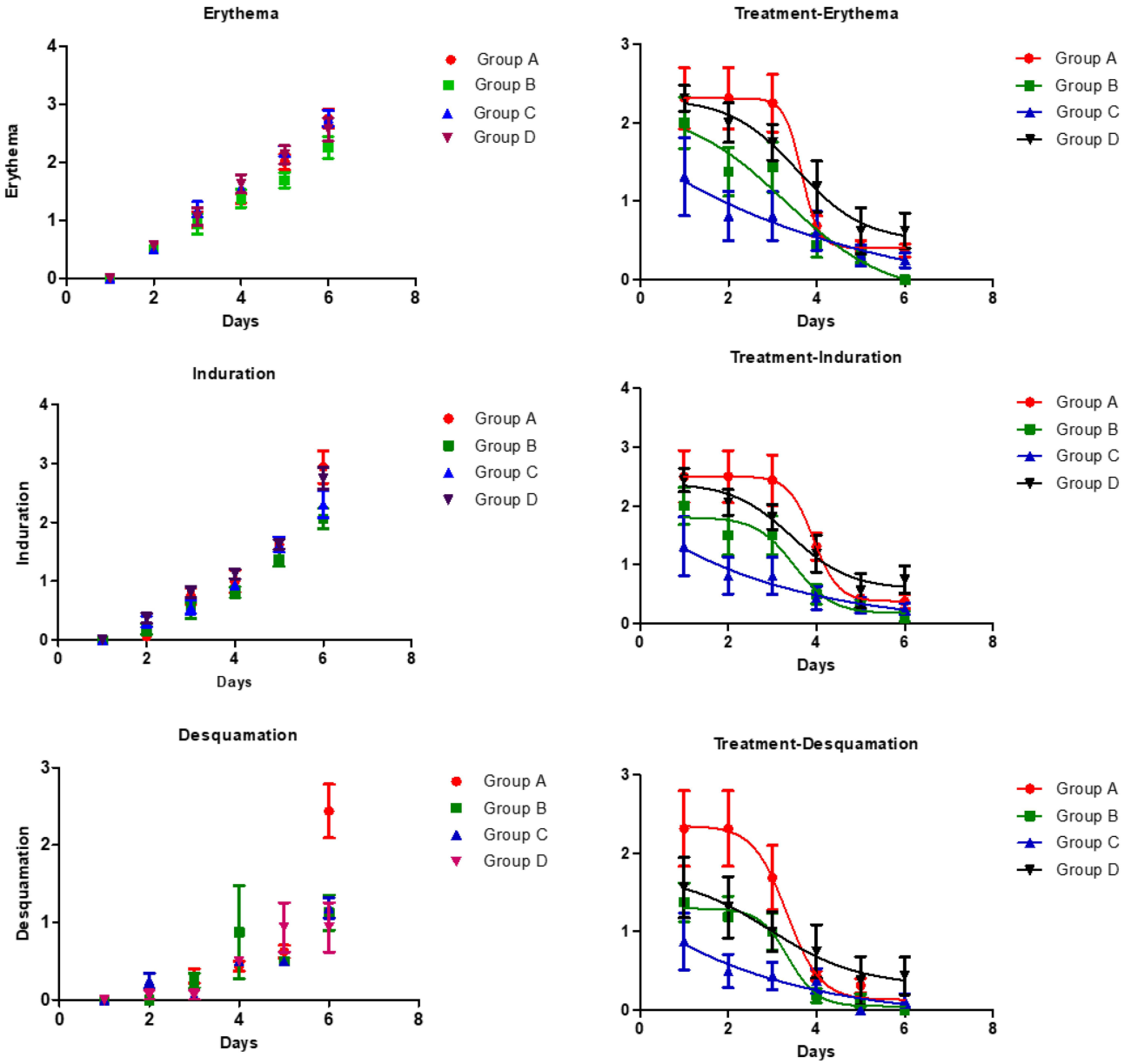

Figure 10 Indikizumab and Secukinumab Inhibit IMIQUIMOD induced psoriasis in mice. Eight to 11 weeks old BALB/c mice received a daily topical dose of 62.5 mg of commercially available IMQ cream (5%) (IMIQUIMOD, from Glenmark) on the shaved back for 6 consecutive days, translating in a daily dose of 3.125 mg of the active compound. Control mice were treated similarly with Vaseline. The animals were treated with 48 μg/mouse/day single dose of antibody. Group A- Control; Group B- Mouse Anti-IL-17A; Group C-Indikizumab; Group D- Secukinumab.

inflammation. To check the effect on chemokine secretion, chemokine CXCL-1 was monitored in the mouse 3T3 cells. Computed value for $LogIC_{50}$ was 1.976± 0.058 pM and 2.26±0.28 pM for Indikizumab and Secukinumab respectively, suggesting that both antibodies are equally potent in inhibiting secretion of CXCL-1. Imiquimod (IMQ), a TLR7/8 ligand and strong immune activator, can cause and worsen psoriasis when applied topically.[33] Hence, mice model of Imiquimod induced psoriasis is a good model to test the potency of Indikizumab. The effect of mouse anti-IL17A, Indikizumab and Secukinumab on IMQ induced psoriasis parameters such as erythema, desquamation and induration on the back of BalbC mice was observed. A comparative analysis indicated that all three antibodies are equally potential in reducing the psoriasis index in the scale of 1–4. However, removal of IMQ also results in reversal of psoriasis plaque on the mice skin. Though there is no effective conclusion on the role and efficacy of the three antibodies, one thing was noticed that reversal of psoriasis skin lesion was immediate after application of antibodies whereas, the same could be noticed only

after 3rd day suggesting that there is some impact of the antibodies. Indikizumab is a human IgG1 variant antibody with similar constant regions of the heavy chain of human IgG1. Hence, its immunomodulating property may be compared to Secukinumab. The humanization of indikizumab aimed to minimize the possibility of immunogenicity when administered to people on a long-term basis. Usually, investigators consider human antibodies are more advantageous compared to humanized antibodies. However, the term "humanized" or "human antibodies" refers only to the process used to create the antibody and has nothing to do with the possibility of immunogenicity in humans.[34] All antibodies have the same overall molecular modules, therefore three CDRs and framework regions (FR) make up the variable region, independent of the antibody's place of origin.[35] Antigen is bound by the CDRs, which are structurally supported by the FR. Since all of the FRs in indkizumab are native antibody sequences for all humans, they are all of human germline origin. Since the most direct interaction of antibody with distinct antigen in this case, human Interleukin-17A, occurs at the CDR regions, it is expected that the amino acids at CDRs should be other than human germline origin. The sequence alignment of Fav regions of Indikizumab, Secukinumab, and Ixekizumab indicates that all three antibodies have different CDRs (Data not shown). Due to their distinct CDRs, therapeutic antibodies both humanized and human can provide patients with yet another therapeutic alternative, but they also carry the risk of immunogenicity.[36,37] Several lines of evidence suggest that IL-17A plays a critical role in the pathogenesis of autoimmune disorders, such as psoriasis, psoriatic arthritis, ankylosing spondylitis, and rheumatoid arthritis, despite its significance in host defense against extracellular bacteria and fungi.[36] In this context, Indikizumab, a same neutralizing class yet novel antibody that may provide an additional option once approved for inflammatory indications.

Conclusion

This program aimed at developing a novel therapeutic for inflammatory indications such as psoriasis, psoriatic spondylitis, and similar inflammatory diseases targeting IL-17A interleukin. The process summarized above concluded that a novel antibody with high target binding affinity and specificity has been developed, and primary characterizations were done to establish proof of concept.

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Author Contributions

All authors took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The Author of this manuscript has no conflict of interest and responsible for all the writing content of the manuscript. Experiments conducted to complete this manuscript were solely with internal resources and funding from the organization. All necessary permissions were taken to conduct the animal experiments.

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