**Discovery and mechanism of ustekinumab**

A human monoclonal antibody targeting interleukin-12 and interleukin-23 for treatment of immune-mediated disorders

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**Abbreviations:** ADCC, antibody-dependent cellular cytotoxicity; CCL, chemokine (C-C motif) ligand; CCR, C-C chemokine receptor type; CXCL, chemokine (C-X-C motif) ligand; CD, cluster of differentiation; CDC, complement-dependent cytotoxicity; CIA, collagen-induced arthritis; CNS, central nervous system; CTLA, cytotoxic T lymphocyte-associated antigen; D, domain; EAE, experimental autoimmune encephalomyelitis; Fab, fragment antigen binding; GMCSF, granulocyte macrophage stimulating factor; Hu-Ig, human immunoglobulin; IFNγ, interferon gamma; IL, interleukin; IL-12R, interleukin-12 receptor complex; IL-23R, IL-23 receptor complex; J, joining; κ, kappa; mAb, monoclonal antibody; NK, natural killer; NKT, natural killer T cells; MS, multiple sclerosis; PsA, psoriatic arthritis; RA, rheumatoid arthritis; ROR, retinoid-related orphan receptor; STAT, signal transduction and activation of transcription; Th, T-helper; TNFα, tumor necrosis factor alpha; Treg, T regulatory; V, variable

Monoclonal antibody (mAb) therapy was first established upon the approval of a mouse antibody for treatment of human acute organ rejection. However, the high incidence of immune response against the mouse mAb restricted therapeutic utility. Development of chimeric, “humanized” and human mAbs broadened therapeutic application to immune-mediated diseases requiring long-term treatment. Indeed, mAb therapeutics targeting soluble cytokines are highly effective in numerous immune-mediated disorders. A recent example is ustekinumab, a first-in-class therapeutic human immunoglobulin (Ig) G, kappa mAb that binds to the interleukins (IL)-12 and IL-23, cytokines that modulate lymphocyte function, including T-helper (Th) 1 and Th17 cell subsets. Ustekinumab was generated via recombinant human IL-12 immunization of hu-Ig transgenic mice. Ustekinumab binds to the p40 subunit common to IL-12 and IL-23 and prevents their interaction with the IL-12 receptor β1 subunit of the IL-12 and IL-23 receptor complexes. Ustekinumab is approved for treatment of moderate-to-severe plaque psoriasis and has demonstrated efficacy in Crohn disease and psoriatic arthritis. The clinical characterization of ustekinumab continues to refine our understanding of human immune pathologies and may offer a novel therapeutic option for certain immune-mediated diseases.

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Monoclonal Antibody Therapies for Immune-Mediated Disorders

The concept of antibodies as therapeutic agents was initially described by Paul Ehrlich, where he reasoned that if a compound could be designed to selectively target a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity.1 Functional and structural characterization of antibodies culminated in several precedent discoveries on the generation and maturation of the humoral immune response.2 The key scientific breakthrough that advanced the evaluation of antibodies as therapeutic modalities was the development of hybridoma technology, which afforded the ability to reliably produce sufficient quantities of “monospecific” or identical antibody moieties, i.e., monoclonal antibodies (mAbs).

The first successful clinical development of a mAb therapeutic agent was a fully mouse anti-CD3 immunoglobulin (Ig) G (muromonab-CD3) for treatment of acute organ rejection.3 However, frequent and significant immune-mediated toxicities were associated with administration of fully mouse mAbs, particularly upon repeated administration. Advancements in genetic engineering resulted in the development of chimeric, humanized and fully human therapeutic mAbs. The reduction or elimination of non-human amino acid sequences resulted in a significant decrease in immune-mediated associated toxicities, which in turn, broadened the potential therapeutic applications.4 Indeed, therapeutic mAbs have become an increasingly important component of pharmacotherapy. It is estimated that more than 300 mAbs are currently in development and, approximately 30 mAbs are approved by the United States Food and Drug
mAbs

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preventing the DNA rearrangement process that is required to assemble a functional mouse antibody heavy chain gene. In addition, the mouse antibody κ light chain and constant region coding sequences were deleted, preventing expression of mouse κ light chains. The human heavy chain “minilocus” of DNA (~80,000 bases in length), which contained coding sequences for four variable (V) regions, sixteen diversity segments, six J segments, IgM constant regions and IgG1 constant regions, were cloned and inserted into the mouse genome. In addition, a human κ light chain “minilocus” of DNA (~450,000 bases in length), containing the coding sequences for at least ten V regions, five J segments and κ constant region, was inserted. These genetic modifications resulted in a mouse strain capable of producing human antibodies in response to immunizations to any antigen of interest (Fig. 2). The human Ig transgenic mouse technology enabled generation of diverse, high affinity, and highly specific mAbs with lower deleterious immunogenicity responses than previously developed rodent mAbs.

To elicit human anti-human IL-12 therapeutic mAbs, the transgenic mice were immunized with human IL-12 antigen. Mice that demonstrated positive serum titers for anti-IL-12 antibodies were selected for hybridoma fusion. Splenocytes, which contain antibody-producing B cells from IL-12 titer-positive mice, were fused with an immortal cell line, and the resulting cell culture supernatants were screened for the presence of anti-IL-12 antibodies by ELISA. Positive clones were then expanded and characterized for their antibody specificity and affinity.

**Interleukin-12 and Interleukin-23 Antibody Discovery and Generation**

Ustekinumab is a human IgG1 kappa (κ) mAb generated by Centocor Research & Development, a division of Johnson & Johnson Pharmaceutical Research and Development, LLC, using human Ig (hu-Ig) transgenic mice obtained from GenPharm, which was subsequently acquired by Medarex and is currently part of Bristol-Myers Squibb of Princeton, New Jersey. In these mice, four distinct genetic modifications replaced the mouse Ig loci with human antibody transgenes. The mouse antibody heavy chain joining (J) coding sequences were deleted, thereby preventing the DNA rearrangement process that is required to assemble a functional mouse antibody heavy chain gene. In addition, the mouse antibody κ light chain and constant region coding sequences were deleted, preventing expression of mouse κ light chains. The human heavy chain “minilocus” of DNA (~80,000 bases in length), which contained coding sequences for four variable (V) regions, sixteen diversity segments, six J segments, IgM constant regions and IgG1 constant regions, were cloned and inserted into the mouse genome. In addition, a human κ light chain “minilocus” of DNA (~450,000 bases in length), containing the coding sequences for at least ten V regions, five J segments and κ constant region, was inserted. These genetic modifications resulted in a mouse strain capable of producing human antibodies in response to immunizations to any antigen of interest (Fig. 2). The human Ig transgenic mouse technology enabled generation of diverse, high affinity, and highly specific mAbs with lower deleterious immunogenicity responses than previously developed rodent mAbs.

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Ustekinumab Mechanism of Action

IL-12 is a heterodimeric cytokine containing two protein subunits named p40 and p35 according to their approximate molecular weight. Subunit binding analysis determined that ustekinumab binds to the IL-12p40 subunit. This was later confirmed by elucidation of the ustekinumab fragment antigen binding (Fab)/IL-12 co-crystal structure. The IL-12 receptor complex consists of IL-12Rβ1 and IL-12Rβ2 chains expressed on the surface of T cells or NK cells (Fig. 4). The IL-12Rβ1 chain binds to the p40 subunit, whereas IL-12p35 association with IL-12Rβ2 confers intracellular signaling. IL-12-mediated signaling includes intracellular phosphorylation of signal transduction activation of transcription (STAT)4 and STAT6 proteins, and functional responses including cell surface molecule expression, NK cell lytic activities and cytokine production, such as IFNγ.

Figure 2. Human antibody transgenic mice. Human heavy and light chain genes were used by GenPharm (later known as Medarex, now part of Bristol-Myers Squibb) to prepare minilocus human immunoglobulin (hu-Ig) transgenic mice (HC4/KCo5). Mice were immunized with human interleukin-12 (IL-12) to produce human antibodies. H, heavy; κ, kappa; ms, mouse.

hybridoma cells were cultured under selection conditions that allowed only hybridoma cells to grow. Growth-positive hybridomas secreting IL-12-specific antibodies were selected for limited dilution subcloning (Fig. 3). Binding and cell-based functional assays using human T cells were utilized to select antibodies that specifically bound IL-12 and inhibited IL-12-mediated responses. A monoclonal hybridoma clone that produced a human IgG1κ antibody capable of binding to and neutralizing human and non-human primate IL-12 was thus identified. The antibody, initially named 12B75, then CNT01275, and later ustekinumab, was chosen for further development based on its superior IL-12 binding and neutralization activity.

As a first step towards preparing a stable cell line producing high quantities of ustekinumab, DNA encoding the entire heavy and light chain genes of the ustekinumab antibody was cloned from the hybridoma cells (Fig. 3). Sequencing of the cloned DNA encoding ustekinumab and their subsequent translation into amino acid sequences, followed by comparison to antibody databases, confirmed that ustekinumab was a human antibody with a human IgG1 heavy chain and a κ light chain. The heavy chain IgG1 constant region sequence is of the G1m (1,3) allotype. The cloned heavy and light chain genes were then introduced into a host cell line by electroporation. Transfected cell lines producing the highest titers of ustekinumab were selected for subcloning and expansion. A single cell line was chosen to support early development. Subsequently, further changes were made to support production using perfusion bioreactors in accordance with Good Manufacturing Practice guidelines, with the resultant recombinant antibody retaining the same amino acid sequence as found in the original hybridoma cell lines. Ustekinumab is purified from the supernatant generated from the bioreactor process.
challenges of the unique dual specificity to the clinical development of ustekinumab have recently been described in detail.\textsuperscript{37}

The p40 subunit of human IL-12 and IL-23 is comprised of three domains (D), i.e., D1–D3, two of which (D2 and D3) are involved in binding IL-12p35 and IL-23p19.\textsuperscript{38,39} Based on a crystal structure of ustekinumab Fab region complexed with human IL-12, the binding epitope for ustekinumab is located in the D1 domain of the p40 subunit, which is spatially distant from IL-12p35 and IL-23p19.\textsuperscript{35} Mutational analysis confirmed amino acid residues within D1 that were required for ustekinumab binding. Through isothermal titration calorimetry analysis, ustekinumab was shown to bind IL-12 and IL-23 equally, with the expected 2:1 antigen-to-antibody stoichiometry. Furthermore, ustekinumab did not bind to structurally related proteins or rodent IL-12/23. Overall, these studies determined the precise specificity and molecular interactions between ustekinumab and IL-12/23p40.

Ustekinumab prevents human IL-12 and IL-23 from binding to the IL-12Rβ1 receptor chain of IL-12 (IL-12Rβ1/β2) and IL-23 (IL-12Rβ1/23R) receptor complexes on the surface of NK and T cells (Fig. 4). This defines the molecular mechanism of action of ustekinumab. Ustekinumab cannot bind to endogenous IL-12 or IL-23 that is already bound to receptor complexes. Thus, ustekinumab is unlikely to mediate Fc effector functions, such as ADCC or CDC. In vitro, ustekinumab will neutralize IL-12-mediated responses, including intracellular phosphorylation of STAT4, cell surface marker expression and IFNγ cytokine production. IL-23-mediated responses are equally inhibited, including intracellular STAT3 phosphorylation and IL-17A, IL-17F and IL-22 cytokine protein production. Collectively, these data demonstrate that by preventing IL-12 and IL-23 from binding to the IL-12Rβ1 receptor, ustekinumab can effectively neutralize human IL-12- and IL-23-mediated cell signaling, activation and cytokine production. It is important to note that while ustekinumab will effectively neutralize IL-12- and IL-23-mediated functional responses, it will not affect immune responses stimulated through other cytokines or cellular activities.

Role of Interleukin-12 and Interleukin-23 in Immune-Mediated Diseases

Studies in animal models and with human disease samples have established a strong link between dysregulation of the Th1/Th17 pathways and dermatologic, rheumatic, gastrointestinal and neurologic disorders, namely psoriasis, RA, Crohn disease and multiple sclerosis (MS). Administration of IL-12 exacerbated disease in murine psoriasis,\textsuperscript{40} chronic colitis,\textsuperscript{51,62} collagen-induced arthritis (CIA) models,\textsuperscript{63} and experimental autoimmune
encephalitis (EAE) models of MS, whereas administration of anti-IL-12/23p40 antibodies is either protective or attenuates disease severity. Subsequent studies in mouse models of EAE and CIA revealed that IL-12/23p40 or IL-23p19 inhibition through genetic ablation or antibody treatment is either protective or attenuates disease severity. In contrast, genetic ablation of IL-12p35 was not protective. Thus, in certain mouse systems, IL-23 mediates many disease pathologies previously attributed to IL-12.

Human genetic and tissue analysis indicates both IL-12 and IL-23 pathways are involved in certain immune-mediated pathologies. However, given the overlap between human Th1 and Th17 pathways and the plasticity between human Th lineages in vivo, it is difficult to distinguish between IL-12 and IL-23 biologies. For example, overexpression of IL-12 was observed systemically or within diseased tissue from a number of human autoimmune/inflammatory disorders. In certain cases, such as MS, protein expression of the IL-12/23p40 in the serum or CNS correlated with disease severity. In addition, gene expression levels of IL-12, IFNγ and IL-23 are elevated in psoriasis skin lesions. Overexpression of both the p35 and p40 subunits of IL-12 are elevated in gastrointestinal tissue of Crohn disease patients and polymorphisms of genes that encode either IL-12/23p40 or the IL-23R are linked to psoriasis, and Crohn disease. In fact, the IL23R R381Q gene variant that protects against psoriasis, Crohn disease and ankylosing spondylitis was recently reported to exert its protective effects through selective attenuation of IL-23-induced Th17 cell effector function, without interfering with Th17 differentiation. Collectively, many published studies support dysregulation of either IL-12, IL-23, or both pathways in human immune-mediated diseases.

**Ustekinumab Clinical Development**

As summarized previously, a strong body of pre-clinical and clinical data established an association between IL12/23p40 and a number of chronic immune-mediated disorders. Of these, psoriasis was chosen as the first-in-human population since it allowed the establishment of proof of concept early in clinical development and afforded the ability to collect and examine diseased tissue for pharmacodynamic effects via minimally invasive procedures. Psoriasis is a chronic immune-mediated skin disorder with significant co-morbidities such as psoriatic arthritis (PsA), depression, cardiovascular disease, hypertension, obesity, diabetes, metabolic syndrome and Crohn disease. Plaque psoriasis is the most common form of the disease and manifests in well-demarcated erythematous lesions topped with white silver scales. Plaques are pruritic, painful and often disfiguring, and a significant proportion of psoriatic patients have plaques on hands/nails, face, feet and genitalia. As such, psoriasis can impose physical and psychosocial burdens that extend beyond the physical dermatological symptoms and interfere with everyday activities. For example, psoriasis negatively impacts familial, spousal, social and work relationships, and is associated with a higher incidence of depression and increased suicidal tendencies.

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**Figure 4.** Ustekinumab mechanism of action. Ustekinumab binds to the p40 subunit of interleukin (IL)-12 and IL-23 and prevents their interaction with the cell surface IL-12Rβ1 receptor, subsequently inhibiting IL-12- and IL-23-mediated cell signaling, activation and cytokine production (image not drawn to scale). NK, natural killer. Adapted from Benson et al.
Histologic characterization of psoriasis lesions reveals a thickened epidermis resulting from aberrant keratinocyte proliferation and differentiation, as well as dermal infiltration and co-localization of CD3+ T lymphocytes and dendritic cells (Fig. 5). While the etiology of psoriasis is not well-defined, gene and protein analyses have shown that IL-12, IL-23 and their downstream molecules are overexpressed in psoriatic lesions, and some may correlate with psoriasis disease severity. Some therapies used in the treatment of psoriasis modulate IL-12 and IL-23 levels, which is speculated to contribute to their efficacy. As illustrated in Figure 5, Th1 and Th17 cells can produce effector cytokines that induce the production of vasodilators, chemoattractants and expression of adhesion molecules on endothelial cells, which, in turn, promote monocyte and neutrophil recruitment, T cell infiltration, neovascularization and keratinocyte activation and hyperplasia. Activated keratinocytes can produce chemoattractant factors that promote neutrophil, monocyte, T cell and DC trafficking, thus establishing a cycle of inflammation and keratinocyte hyperproliferation.

Results of three Phase 3 clinical studies of ustekinumab in the treatment of moderate-to-severe plaque psoriasis have been published. Ustekinumab administered by subcutaneous injection at weeks 0 and 4 and then once every 12 weeks exhibited rapid and sustained clinical response, as assessed by the Psoriasis Area and Severity Index, a validated efficacy tool for psoriasis. A Phase 3 study comparing ustekinumab with etanercept, a TNF antagonist, demonstrated that the efficacy of ustekinumab was superior to that of etanercept over a 12-week period in patients.
with moderate-to-severe psoriasis. In two Phase 3 clinical studies, PHOENIX I and PHOENIX II, ustekinumab exhibited a half-life of approximately 3 weeks. Immune response rates against ustekinumab ranged from 3 to 5%. In addition, reported adverse events were relatively mild, with the majority of events including susceptibility to mild infections such as nasopharyngitis and upper respiratory tract infection. Rates of infection were not higher in ustekinumab-treated patients when compared with placebo-treated patients over 12 weeks of therapy; nor were they increased in association with higher, relative to lower, ustekinumab doses. Also, rates of serious infections, cardiovascular events, injection site reactions and malignancies were low. Taken together, the clinical observations of ustekinumab in psoriasis have supported its first-in-class status and confirmed the fundamental role of IL-12 or IL-23 in psoriasis pathogenesis.

Completed ustekinumab Phase 2 studies in Crohn disease and PsA indicate that blockade of IL-12/23p40 also results in clinical response in these diseases. Ustekinumab treatment resulted in significant attenuation of arthritis signs and symptoms of PsA in addition to diminishment of psoriatic plaques. The safety and efficacy of ustekinumab in PsA is currently being evaluated in a Phase 3 study. Ustekinumab was also recently shown to induce and maintain clinical response in patients with moderate-to-severe Crohn disease who had previously failed one or more TNF-antagonist mAbs. The efficacy and safety of ustekinumab in moderate-to-severe Crohn disease are currently being further evaluated in three Phase 3 studies. These clinical observations suggest that psoriasis, PsA and Crohn disease share common pathological immune pathways, which include IL-12 and IL-23 (Fig. 6).

The discordance between animal model causality and human disease association of IL-12/23 and the ustekinumab clinical trial results in MS is not well understood.
New indications for ustekinumab are also being explored. One example is sarcoidosis, which is a chronic, heterogenic and multi-systemic granulomatous disease of unknown cause. Release of cytokines such as TNFα and IL-12 during the formation of sarcoid granulomas and upregulation of IL-12 in lung tissue are reported in patients with pulmonary involvement.2 However, the role of IL-12 or IL-23 in the development of cutaneous sarcoid lesions is not yet clearly elucidated. Genes linked with the Th1 pathway, as well as expression of IL-23 and IL-23R, are associated with cutaneous sarcoidosis.80 In fact, gene expression of IL-12/23p40 was comparable or higher, than levels observed in psoriatic skin lesions. The effect of ustekinumab on granuloma formation in sarcoidosis is currently being assessed in a Phase 2 proof of concept study.81 Collectively, observations to date from clinical studies with ustekinumab suggest common immune pathways between psoriasis, PSA and Crohn disease, with the role of IL-12/23 in sarcoidosis under evaluation (Fig. 6).

Summary

Ustekinumab is a “first-in-class” anti-IL-12/23p40 mAb approved for the treatment of moderate-to-severe plaque psoriasis and is one of the first approved therapeutic mAbs generated directly through hu-Ig mice technology with no further molecular engineering. The mAb binds to the p40 subunit of both IL-12 and IL-23, preventing the interaction of both cytokines with the IL-12Rβ1 subunit that is common to both IL-12 and IL-23 cell surface receptors. Ustekinumab prevents IL-12- and IL-23-mediated downstream signaling, gene activation and cytokine production. Ustekinumab exhibits a long biologic half-life and low immune response rate, which translates into 12-week dosing intervals for treatment of moderate-to-severe psoriasis. The positive clinical results of ustekinumab observed in psoriasis and other immune-mediated disorders, such as Crohn disease and PSA, indicate that Th1 or Th17 lineages play a critical role in the underlying pathologic processes of these immune disorders. Similar to the TNF antagonists, ustekinumab further demonstrates that mAb-directed cytokine targeting can effectively attenuate cytokine-mediated pathologic processes, presumably through altering the local cytokine environment within diseased tissues. The relative roles of IL-12 and IL-23 in immune pathologies are not clearly defined and would require further clinical evaluation with agents specifically targeting the individual cytokines.

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Notes

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