Innovations in receptor-targeted precision imaging at Navidea: diagnosis up close and personal

Navidea Biopharmaceuticals envisions a world where disease can be precisely identified and treated. Today, faced with a multitude of illnesses, millions of people are left with diagnostic uncertainty. As patients and their physicians seek accurate diagnosis of their condition, precision diagnostics are playing important new roles in medicine. Navidea is a global leader in precision diagnostics that can pinpoint molecular sites and pathways that identify the presence and status of disease. Through our innovative and flexible platforms and products, we are committed to improving diagnostic accuracy, empowering clinical decision-making, and enhancing patient care. This paper, written in cooperation with our research collaborators, addresses specific diagnostic questions in diseases where there is significant unmet medical need for such innovation. It pays particular attention to elements of the inflammatory process in multiple disease states and focuses on targeting macrophages as an underlying contributor to the disease progression process.

Inflammation-focused precision diagnostics

Inflammation is a necessary response to pathogenic invasion, wound healing and tumour expression, and is an integral process in a host of other diseases as well. We now realize that this inflammatory process is remarkably complex and can be driven by macrophages, with as many integrated and highly choreographed elements as a precision watch. However, in many disease states or maladies — including autoimmune diseases (such as multiple sclerosis, diabetes, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Crohn’s disease), infectious diseases (HIV and tuberculosis), tumorigenesis, neurodegenerative disorders (dementias) and cardiovascular disease (vulnerable plaque and atherosclerosis) — the presence of the macrophage becomes a self-winding component of the progression of the disease state. Table 1 lists a number of these diseases and their impact on populations with regard to their incidence and/or prevalence in the United States and worldwide.

Macrophages are part of the response to cell damage resulting from infection or injury. During this process, local cellular or plasma-derived factors encourage macrophage cell types to migrate to the affected tissue areas. These immune cells increase their expression of inflammatory cytokines, recruiting additional immune cells to resolve the damage. However, in localizing to the sites of the disease process, macrophages (or their tissue-specific counterparts, such as microglial cells of the brain) can become

Figure 1 | Targeting macrophages via CD206, the mannose receptor. A. CD206 structure. B. The structure of tilmanocept showing the CD206–exploiting mannose moieties.
one of the ‘self-winding’ elements intrinsic to the pathobiology of a mounting disease state. Components of the macrophage-driven disease mechanism, rather than acting as biological rheostats that bring the aberrant state back under control, can become mechanistically dysregulated and accelerate or enhance disease progression. The potential for dysregulation and induction by macrophages to various pathological and autoimmune conditions is an area of increasing focus in medicine. The number of people affected by all the inflammatory diseases combined is estimated at more than 40 million in the United States and perhaps 700 million worldwide (Table 1), making these macrophage-mediated diseases an area of remarkable clinical importance.

The involvement of the macrophage in the manifestation of disease provides an opportunity to exploit the characteristics of macrophage-driven pathologies. One elegant approach to targeting macrophages involves a key receptor of the macrophage, CD206, the human mannose receptor. The CD206 receptor has been successfully exploited as a target of precision diagnostics, for example using the novel molecular compound tilmanocept, which locks into CD206 through its mannose moieties (Fig. 1), and is then taken into the macrophage where it persists in stable, non-digesting vesicles.

Such a precision targeting mechanism presents itself as a novel pathway to identify the locality of disease and the key functions of the macrophage-driven disease process. The development of agents designed to detect such pathology has lagged behind those for imaging general anatomic features, in part because of the lack of a specific targeted receptor binding system. The exploitation of the CD206 receptor serves as an entry point to identifying underlying pathology and hence to the entire self-winding inflammatory process.

Exploiting CD206 raises the possibility of using multiple reporter types, including isotopes such as technetium-99m, fluorescent molecules, photo-activated molecules, and pharmaceuticals or toxins.

Below, we discuss the opportunities for innovative diagnostic imaging and the ability to use a targeting approach through the CD206 receptor to identify underlying macrophage-associated inflammation as an element of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), tuberculosis (TB) and in Kaposis sarcoma (KS)/HIV infection, all disease states that express unique biological features involving macrophages as key elements of the activation and progression of the inflammatory disease state.

These disease states, as well as others listed in Table 1, all have macrophage involvement and associated CD206 expression. Indeed, they provide opportunities for the exploitation of CD206 as a key portal for imaging pathological states where such clinical data provide the potential for new and more focused clinical decision-making and therapeutic choices.

These discussions represent a broader and more common theme involving macrophage-mediated pathology, and we anticipate that initiatives in these areas will produce positive results with improved potential for diagnostic evaluation and therapeutic drug development.

In short, macrophage-involved diseases provide opportunities to demonstrate the concept of using a CD206-targeting platform to pinpoint the presence and status of disease in a range of disorders, and suggest that a precision diagnostics approach to targeting the macrophage may be fruitful in enhancing diagnostic accuracy, clinical decision-making and, ultimately, patient care.

**Rheumatoid arthritis and systemic lupus erythematosus**

Both rheumatoid arthritis and systemic lupus erythematosus have been the subject of intense study. Most researchers have focused on the role of the adaptive immune system in these diseases and have uncovered significant abnormalities. However, an emerging body of research suggests that the innate immune system also plays a significant role in the active chronic inflammatory and prodomal phases of RA and SLE. This assertion is supported by data suggesting that infections are possible triggers of RA and SLE, as these same infections strongly activate the innate immune system. Clearly, these and many other environmental factors (such as smoking) have a significant impact on the innate immune system, and may predispose patients to RA and SLE.

These types of observations raise the possibility that the innate immune system may be more than a collaborator in the complex pathogenesis of RA and SLE — such factors may be the instigator of the initial events that lead to clinically recognized disease. This view of the immune system has tissue macrophages, in conjunction with permissive genes, as central to the pathogenesis.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Incidence</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaposi’s Sarcoma</td>
<td>2</td>
<td>3,700</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>11</td>
<td>21,000</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>260</td>
<td>24,000</td>
</tr>
<tr>
<td>Crohn’s Disease</td>
<td>630</td>
<td>2,000</td>
</tr>
<tr>
<td>Systemic Lupus</td>
<td>1,500</td>
<td>60,000</td>
</tr>
<tr>
<td>Erythematosus</td>
<td></td>
<td>33,000</td>
</tr>
<tr>
<td>Rheumatoid</td>
<td></td>
<td>122,000</td>
</tr>
<tr>
<td>Arthritis</td>
<td></td>
<td>480,000</td>
</tr>
<tr>
<td>Neurodegenerative Diseases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
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<tr>
<td>Atherosclerosis</td>
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*Table 1 | Macrophage-Associated Diseases for CD206 Targeting (in thousands)*
of an autoimmune disease. As described in the introduction, this model suggests that macrophages receive environmental, infectious and hormonal signals, and as a result are transformed into inflammatory cells that are critical in the prodromal phase of autoimmunity (Fig. 2). Because of the length of the prodromal phase in SLE and other autoimmune diseases, there is an opportunity to develop predictive biomarkers that can accurately inform and guide clinical decision making. This gives physicians the opportunity for targeted interventions that could restore immune-system homeostasis and prevent the full development of clinical disease.

The major obstacle for such approaches at present is the lack of data regarding early or prodromal events in autoimmune diseases and the involvement of macrophages. There is a great need for more studies in RA and SLE that focus on identifying abnormalities in macrophage differentiation where the elements of the inflammatory process can be used as early biomarkers of autoimmune disease.

The inability to localize macrophage and monocyte function in vivo is the barrier to tracking the progression of RA and SLE. However, the recent development of tilmanocept is a major step in engineering the tools to examine the role of macrophages in various autoimmune diseases. Tilmanocept, by binding the human mannose receptor (CD206) on the surface of macrophages, can assist in identifying these cells both in vitro and in vivo (Fig. 2). Ongoing research is examining the presence of macrophages in the synovial fluid of patients with RA, using fluorochrome-labelled tilmanocept, and future studies will use tilmanocept to examine tissue macrophage distribution in RA patients. The feasibility of this approach was recently demonstrated in an animal model of RA through the localization of macrophages in vivo using radiolabelled nanobodies that target the murine mannose receptor. This was also demonstrated in a similar model using the fluorescent-labelled tilmanocept-Cy3 (Fig. 3). These studies have the ultimate goal of examining the mannose receptor (CD206) distribution using tilmanocept as a predictive biomarker in the prodromal phase of RA.

In SLE, the presence of activated tissue macrophages in the kidney has been established as a marker of disease onset and disease remission in lupus nephritis. Exploring the possibility of using tilmanocept and nuclear imaging as a means for the evaluation of lupus nephritis activity without the need for repeated kidney biopsies is an appropriate experimental approach. Future efforts will seek to determine whether there is a positive correlation between histologic examination of kidney tissue and the reporter molecule-labelled tilmanocept scan. Future clinical studies could also examine the pathologic presence of tissue macrophages in vivo in other organs using reporter molecule-labelled tilmanocept and correlate the results with other diagnostics. Other areas that may be useful to consider include stratification of disease severity and identification of early organ involvement.

Through innovative targeting of the CD206 receptor (for example, with tilmanocept), physicians may gain new diagnostic tools in the evaluation and treatment of patients with autoimmune diseases. The use of such an approach may also improve the identification of patients who are in the prodromal phase for RA or SLE, which could cause a paradigm shift in the diagnosis and management of these diseases from limiting tissue injury to prevention.

Tuberculosis

Infectious diseases account for more than 25% of human deaths in the world annually and are currently the third leading cause of death in the United States. As infectious diseases remain significant health problems around the world, a central component of the effort to eliminate or thwart these diseases will rest on having markedly improved imaging and diagnostic capabilities, as well as more effective therapies and vaccines. The lack of accessibility of many diagnostic — particularly precise diagnostic imaging — and chemotherapeutic agents to infected or diseased tissue sites in patients with a variety of infectious diseases remains a formidable obstacle to effective cures. This is especially true for chronic and recurring infections caused by intracellular pathogens of macrophages. Despite the rapid development of medicinal and drug delivery technologies, the targeting of drugs to immune cells, especially macrophages, for effective diagnosis and treatment of the underlying diseases has not been addressed adequately. Attention is being paid to the mannosylation of imaging agents such as tilmanocept for advanced imaging and diagnostic strategies that optimize CD206-mediated uptake by macrophages in target tissues during infection. It is anticipated that such strategies will enable the use of smaller amounts of drugs for optimal clinical effects, thereby increasing efficacy and reducing toxicity.

As noted previously, the CD206 human mannose receptor (Fig. 1) contains extracellular regions that include a cysteine-rich domain that binds glycoproteins bearing sulphated sugars that terminate in 4-0-SO-linked glyco- proteins as well as pituitary hormones from the circulation. Because many pathogenic microbes are coated with mannose-containing structures, macrophage CD206 interacts with those pathogens in a form of the host molecular mimicry. The CD206 recognizes a variety of microorganisms including bacteria, fungi, viruses and parasites. Most of the CD206-binding organisms are intracellular pathogens. Because CD206 is an abundantly expressed, rapidly recycling receptor, targeting it is an attractive and viable strategy for the delivery of imaging and diagnostic agents.

Tissue macrophages, which are the main patrolling immune cell of the innate immune system and a communication cell for the development and maintenance of the adaptive immune system, represent a highly diverse population in terms of phenotype and function, depending on their tissue site and state of activation. They have been variably termed M1, M2 and immunoregulatory, for example, highlighting their spectrum of activities (Fig. 2). Alveolar macrophages
are prototypic alternatively activated macrophages and express abundant CD206, which mediates interaction with various airborne pathogens. In the cancer tissue microenvironment, tumour-associated macrophages (TAMs) have been described that play important roles in tumour invasion, proliferation and metastasis. These are M2-type macrophages and express high levels of CD206. Studies in cancer research have targeted CD206 expressed on TAMs for the purpose of delivering imaging, diagnostic agents to tumour sites. Therefore TAMs represent an appealing biological target for cancer imaging and diagnosis. The successful optimization of techniques for efficient CD206-mediated delivery of imaging and therapeutic agents set the stage for targeting CD206-expressing macrophages for similar clinical applications in infectious diseases including TB (Fig. 4).

Tuberculosis is a respiratory infection caused by the bacterial pathogen *Mycobacterium tuberculosis*. It currently infects approximately one-third of the world’s population and is the second most common cause of death from an infectious agent worldwide. The ability to rapidly and specifically image TB lesions for the purpose of diagnosis in active TB patients remains limited, however. A histopathologic hallmark of the host response to TB is the granuloma, a unique microenvironment that in most cases sustains *M. tuberculosis* for decades with no apparent clinical symptoms. There is no method for accurately imaging granulomas in patients with latency, a condition in which treatment can reduce the risk of developing active TB.

The *M. tuberculosis* bacterium is a prototypic intracellular pathogen of macrophages where such macrophages play a major role in both latent and active TB. Macrophages are essential for granuloma formation and maintenance. The granuloma is where *M. tuberculosis* is both controlled and allowed to persist, yet this unique environment remains one of the least understood aspects of the host–pathogen relationship. What is widely recognized, however, is that the granuloma microenvironment represents a formidable barrier to the delivery of diagnostic agents, akin to the tumour microenvironment, and some parallels can be drawn, including physiological barriers, such as reduced oxygen tension and altered phenotype, and the function of macrophages.

Targeting macrophage CD206 is an attractive strategy for the imaging and diagnosis of TB. Schlesinger et al. discovered the role of CD206 in the uptake of *M. tuberculosis* by human macrophages 20 years ago, and more recently revealed the role of CD206 in regulating macrophage responses to this pathogen. To date, there has been no report on the systematic evaluation of CD206 on macrophages present in hypoxic conditions within TB granulomas akin to TAMs. However, it is likely that such macrophages express CD206 as well as CD163. CD206 has been implicated in macrophage adhesion and fusion during granuloma formation. In addition, peroxisome proliferator-activated receptor-γ (PPAR-γ) mediates the induction of CD206 and foam cells, the latter being found in granulomas. PPAR-γ is upregulated by *M. tuberculosis* engagement of CD206, which could potentially help to sustain the regulated inflammatory environment inside granulomas. Taken together, it is plausible that macrophages expressing CD206 within TB granulomas can serve as an effective imaging and diagnostic target.

So targeting of CD206 by tilmanocept and derived compounds has the potential to enhance imaging, particularly in TB granulomas. Based on human data, the localization of tilmanocept is highly specific and is concentrated on CD206-expressing cells, leading to the identification of only macrophage-involved tissue; tissue expressing little to no CD206 produces virtually no signal (Fig. 5). Innovative strategies for targeting the CD206 pathway for the rational design and effective delivery of imaging agents into uniquely located tissue macrophages, particularly with the readily available product tilmanocept, holds promise for the diagnosis and treatment of a variety of infectious diseases.

**Figure 4** Schematic diagram showing macrophage phagocytosis of *Mycobacterium tuberculosis* through CD206 and specific binding of tilmanocept alone, conjugated to drug derivatives or coating encapsulated drug (in liposomes, nanoparticles, etc.). Because CD206 is a rapidly recycling receptor, tilmanocept and drugs are efficiently delivered to the cells where they encounter microbes in the cytosol or in phagosomes (P, phagosome; L, lysosome).

**Figure 5** Co-localization of CD206 and tilmanocept on macrophages. Confocal microscopy images show CD206 (MR) expression (green in top left), tilmanocept binding by the macrophage (red, top right), and co-localization between CD206 and tilmanocept in both confocal and phase-contrast images (yellow in bottom images).

**Kaposi’s sarcoma**

The pathogenesis of cancer is a complex multistep process involving the acquisition of genetic abnormalities by cancer cells and tumour progression associated with an ongoing inflammatory process. As described earlier, the inflammation is generally driven by TAMs, and these cells have been proven in a variety of systems to provide a supportive environment for tumour progression. In addition, there is increasing evidence that tumour metastasis is associated with tumour cells that co-express macrophage markers that allow more efficient metastasis. As metastasis is critical to the overall pathogenesis of cancer and survival in patients with cancer, both TAMs and tumour cells bearing macrophage markers would be important targets for quantitative imaging in the development of anti-cancer therapeutics. In this context, HIV-associated KS is a form of cancer in which inflammation plays a critical role in tumour development. Because tilmanocept binds to CD206 (Fig. 1a,b), it could have a significant role in the development of a new wave of diagnostic and anti-tumour agents directed against TAMs and metastatic tumour cells by imaging their metastatic pattern and response to therapy. A model that depicts a possible KS pathogenesis paradigm is shown in Fig. 6.

**Macrophage function**

Macrophages are one of the most versatile cell types in the body, largely owing to their ability to fluctuate between activation states...
Table 2 | Immunophenotypic analysis of Kaposi’s sarcoma (KS) from patients, showing the proportion of tumours that express macrophage antigens on tumour-associated macrophages and tumour cells.

<table>
<thead>
<tr>
<th>Marker</th>
<th>MAC387</th>
<th>CD163</th>
<th>CD68</th>
<th>CD206</th>
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</thead>
<tbody>
<tr>
<td>Negative</td>
<td>6.0%</td>
<td>15.2%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Macrophage only</td>
<td>19.6%</td>
<td>12.1%</td>
<td>9.8%</td>
<td>8.9%</td>
</tr>
<tr>
<td>Macrophage and KS spindle cells</td>
<td>74.2%</td>
<td>72.7%</td>
<td>90.2%</td>
<td>91.1%</td>
</tr>
</tbody>
</table>

Table 2 | Scheme showing the potential evolution of Kaposi’s sarcoma (KS) tumour cells as CD206-positive hybrid endothelial cell and macrophages. This mechanism shows how KS spindle cells may express macrophage antigens. Macrophages make up a large component of KS lesions (a). Macrophages are highly fusogenic cells that may form fusion hybrids with surrounding KS spindle cells. The result would lead to co-expression of both KS and macrophage antigens (b). Most KS lesion-associated cells express CD206 and will be imaged by tilmanocept (c).

**HIV-related KS**

AIDS-related KS is an aggressive, multifocal, angioproliferative neoplasm associated with Kaposi’s sarcoma herpesvirus (HHV8/KSHV) infection. It involves cutaneous and visceral tissues, with later forms of disease associated with widespread organ involvement. It is the most common cancer in patients infected with HIV. Effective antiviral therapy has produced a decline in the incidence of AIDS-related KS, but HIV-infected individuals still have a 3,640-fold greater risk of developing KS than the uninfected population. In general, no imaging studies have been able to identify specific KS-involved tissues, apart from standard ultrasound and CT imaging, in which therapy-associated changes are implied to be associated with KS lesion shrinkage.

**Macrophage antigens and KS**

Kaposi’s sarcoma lesions are comprised of KS spindle cells infected with HHV8/KSHV, as well as numerous macrophage antigen-expressing cells. Crucially, a large collection of both skin and visceral forms of KS were tested to determine whether the CD206 molecule would be present on both KS tumour cells and TAMs, allowing the potential for using tilmanocept as a tumour-specific imaging agent capable of identifying both tumour cells and TAMs in patients with KS. Historically, no imaging platform has been able to identify KS-specific lesions in patients with KS. This has caused problems for the delivery of clinical care, as physicians are unable to appropriately stage patients with KS, other than by tracking skin lesions. KS is known to involve lymph nodes and organs, but to date no approach has been able to confirm tumour involvement beyond skin. Our investigations have confirmed that the majority of both TAMs and KS cells express the macrophage marker CD206, the receptor for the tilmanocept imaging agent.

The observation of tilmanocept binding specificity to CD206-positive macrophages noted above (Fig. 5) has been extended to CD206-positive tissue from KS patients (Table 2).

This performance makes tilmanocept an ideal agent for use in patients with KS to stage and quantitatively image tumour-specific response to therapy. By extension, other classes of tumours may be similar hybrid-like cells and may be imaged with tilmanocept and clinically addressed using macrophage-targeted therapy.

**Our vision**

Navidea’s innovative precision diagnostics are focused on addressing unmet needs to benefit individuals — patients, families, physicians and caregivers — touched by devastating conditions such as cancer, inflammation-mediated disorders and neurodegenerative diseases. The vision is to bring new ideas in medicine and scientific discovery to the people who need it most.