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Development of optical probes for in vivo imaging of polarized macrophages during foreign body reactions

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ABSTRACT

Plasticity of macrophage (MΦ) phenotypes exist in a spectrum from classically activated (M1) cells, to alternatively activated (M2) cells, contributing to both the normal healing of tissues and the pathogenesis of implant failure. Here, folate- and mannose-based optical probes were fabricated to simultaneously determine the degree of MΦ polarization. In vitro tests show the ability of these probes to specifically target M1 and M2 cells. In an in vivo murine model, they were able to distinguish between the M1-dominated inflammatory response to infection and the M2-dominated regenerative response to particle implants. Finally, the probes were used to assess the inflammatory/regenerative properties of biomaterial implants. Our results show that these probes can be used to monitor and quantify the dynamic processes of MΦ polarization and their role in cellular responses in real time.

1. Introduction

In the process of wound healing responses, macrophages (MΦs) first become activated to destroy the potential pathogen and subsequently launch regenerative responses to restore tissue homeostasis. Unfortunately, the continuous presence of many medical implants may contribute to a long-term overstimulation of MΦs, leading to chronic inflammation and poor wound healing. This unbalanced reaction, also known as the foreign body response, often leads to implant failure due to the formation of a cellular, protein-mediated capsule which impedes the function of the implant [1–3]. In recent years, increasing knowledge has revealed a link between the contradictory activities and polarization of MΦs [4]. Most commonly, these are grouped into classically activated (M1) cells which are pro-inflammatory in nature, or alternatively activated (M2) cells which are regulatory in nature. These cells exert almost opposite effects on the adaptive immune response, triggering either tissue destruction or regeneration [4,5]. Previous results have shown that MΦ polarization has a profound impact on tumorigenesis, immune responses and angiogenesis [6–10]. Studies have also confirmed that both classically activated and alternatively activated MΦs can alter tissue response through the degree of activity and cytokine production in models such as pulmonary fibrosis, metastatic disease and infectious disease [8,9,11–13]. There is, however, a dearth of information on the relative degree of MΦ polarization that leads to altered destructive/regenerative responses. This scarcity may partly be due to the lack of methods to monitor and quantify the relative polarity of MΦs in real time.

Mounting evidence supports the hypothesis that, via a plethora of receptors, polarized MΦs are able to respond to different signals in the physiological milieu. In fact, some of these receptors have been used to distinguish between different subsets of polarized MΦs. The folate receptor, for instance, has been shown to be up-regulated and specific for MΦs activated by an inflammatory stimulus [14]. The folate receptor, in addition to being expressed in the kidney and placenta, is also up-regulated in many malignant tissues such as ovarian, breast, bronchial and brain cancers. However, other normal tissues express only low or undetectable levels of folate receptor [15,16]. Thus folate receptors have been the target of several delivery systems for therapeutic drugs, and imaging agents. In a study collecting murine MΦ after peritoneal lavage, it was found that only the activated MΦ subset and not the resident MΦs, granulocytes, lymphocytes or erythrocytes,

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expressed up-regulated folate receptors [14]. In addition, these folate-receptor-positive MΦs also produced reactive oxygen species (ROS) and expressed tumor necrosis factor-α (TNF-α) as well as surface markers for classically activated M1 MΦ. Low levels of surface markers were detected for alternatively activated MΦs [14]. Similarly, the folate receptor has been identified on activated synovial MΦs in rheumatoid arthritis [15] and in MΦs in the pathogenesis of atherosclerosis [16]. We have previously developed a folate receptor-targeting probe to quantify the degree of inflammatory responses around a medical implant. This probe was shown to have high affinity for lipopolysaccharide (LPS)-activated MΦs in vitro and LPS-induced inflamed tissue in vivo [17]. Furthermore the folate-receptor-targeting probe was able to detect activated MΦs surrounding biomaterial implants and assess the overall inflammatory reaction to subcutaneous implants [17].

Many recent studies have used the mannose receptor as a target ligand for M2 alternatively activated MΦs. The mannose receptor is an important endocytic receptor which provides a clearance system for molecules up-regulated during inflammation such as tissue plasminogen activator, myeloperoxidase, thyroglobulin and some microbial ligands [18,19]. The mannose receptor is expressed by MΦs and selects endothelial cells but not monocytes or neutrophils [19]. Although activated dendritic cells (DCs) have limited expression of mannose receptor, most of the DCs do not constitutively express mannose receptor in vivo [18]. The mannose receptor of MΦs can be substantially up-regulated by stimulation with interleukin (IL)-4, IL-10 and IL-13, although the activation pathways may be different among cytokines [18–20]. Coincidentally, expression of mannose receptor is diminished by interferon-γ stimulation, which is a hallmark initiator of M1 responses [19]. The contrast between the folate receptor and the mannose receptor may therefore provide a vital difference in the detection of polarized MΦs in the context of biomaterial-mediated inflammation and resolution. We therefore designed analogous imaging probes coupled with distinct near-infrared (NIR) indicators to simultaneously monitor the dynamic process of MΦ polarization around biomaterial implants.

The following study was aimed at fabricating distinct folate-receptor- and mannose-receptor-targeting probes which can be used simultaneously to identify and quantify the degree of MΦ polarization in vivo. Specifically, folate- and mannose-conjugated NIR probes were fabricated by covalently linking ligands with a NIR dye-labeling polyethylene glycol (PEG) platform. For simultaneous imaging, folate was linked to Oyster800 dye (emission: 796 nm) and mannose was linked to Oyster680 dye (emission: 693 nm). The efficiency with which the imaging probes recognize activated M1 or M2 cells, as well as the toxicity of these probes, was then assessed in vitro. The applicability of the probes to in vivo imaging was then investigated using BALB/c mice and subcutaneous implantation of poly(lactic acid) (PLA) particles. In a model of infection, some of these particles were mixed with either LPS or *Staphylococcus aureus* bacteria to investigate the ability of the probes to monitor infection-related complications and the resolution of such responses. By comparing NIR probe fluorescence intensities and histological evaluation, we explored the possibility of using folate- and mannose-based probes to monitor and quantify the extent of such reactions. Finally, using a well-established particle implant model, we determined the ability of the probes to assess the resolution of the inflammatory response to various biomaterials by imaging M1 and M2 in vivo. The ability to simultaneously monitor both M1 and M2 responses and the dynamic effects of these cells in real time may greatly improve our understanding and lead to enhanced methods to evaluate and diagnose implant safety and performance.

2. Materials and methods

2.1. Materials

Both linear NH_2-PEG-COOH (M_w = 5000) and linear t-BOC-PEG-NH_2 (M_w = 5000) were purchased from JenKem Technology USA Inc. (Allen, TX). Oyster®-800 TFP ester (Oyster800) and Oyster®-680 TFP ester (Oyster680) were purchased from Boca Scientific Inc (Boca Raton, FL). Folic acid, 4-aminophenyl α-D-mannopyranoside (mannose), *N*-hydroxysuccinimide (NHS), *N*,*N*′-Dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-(dimethlaminopropyl) carbodiimide (EDC), and all other chemicals were purchased from Sigma–Aldrich (St Louis, MO).

2.2. Preparation of folate- and mannose-based probes

Both folate- and mannose-based probes were fabricated using PEG linear polymer as a carrier with one ligand and one fluorophore per probe. The folate-based probe was prepared as described in a previous publication with minor modification [21]. Briefly, folic acid (FA, 50 mM) was dissolved in dimethyl sulfoxide (DMSO), and then DCC (100 mM) and NHS (100 mM) were added to the above folic acid solution. The mixture was incubated overnight at room temperature, and then the byproduct 1,3-dicyclohexylurea was removed by centrifugation. The above-prepared activated folic acid (80 mM) solution was mixed with t-BOC-PEG-NH_2 (4.0 mM) and the mixture was incubated for 24 h at room temperature to couple folic acid to the amine group of t-BOC-PEG-NH_2 (t-BOC-PEG-FA). This intermediate was dialyzed exhaustively against DMSO and then against deionized (DI) water (Spectra/Por® 1-7 Regenerated Cellulose Membrane, cutoff:3.5K, Spectrum Laboratories Inc.). The dried intermediates were dissolved in dichloromethane and then treated with trifluoroacetic acid to cleave t-BOC groups (NH_2-PEG-FA). After purifying against DI water and lyophilizing, the folate-based probe was prepared by incubating Oyster800 TFP ester and NH_2-PEG-FA (molar ratio 1.5:1) in PBS buffer (pH 8.2) for 24 h at room temperature. The unconjugated dye was removed by dialysis against DI water until undetectable by UV–visible spectrometer and/or fluorometer. The obtained probe was freeze-dried and stored at 4 °C for further use.

For preparation of the mannose-based probe, a similar method was carried out using an established EDC procedure [22,23]. Briefly, Oyster680 TFP ester and NH_2-PEG-COOH (molar ratio 1.5:1) were incubated in PBS buffer (pH 8.2) for 24 h at room temperature to obtain Oyster680-PEG-COOH. The Oyster680-PEG-COOH was dialyzed against DI water until no free dye was detectable in dialysate solution by UV–visible spectrometry and/or fluorometry. After freeze-drying, EDC was added to mannose and Oyster680-PEG-COOH PBS buffer (pH 4.8) (molar ratio Oyster680-PEG-COOH:EDC:mannose = 1:35:30). The mixture solution was incubated for 24 h at room temperature to obtain the mannose-based probe. The probe was purified thorough dialysis and freeze-dried for further use. Chemical structures of both folate- and mannose-based probes were characterized using a Nicolet 6700 FT-IR spectrometer (Thermo Nicolet Corp., Madison, WI). The optical properties of the two probes were analyzed using a microplate reader (Infinite® M200; Tecan Group Ltd., Mannedorf, Switzerland). To normalize fluorescence intensity from the plate reader the highest peak intensity was taken to be "100". Based on nuclear magnetic resonance measurements, the folate/PEG and mannose/PEG conjugation efficiencies were >90%, which concurs with previous work [24].

2.3. Cell isolation and culture procedures

Primary murine MΦs were obtained as previously described [25,26]. Briefly, the bone marrow from the femur and tibia of