Folate-functionalized dendrimers for targeting *Chlamydia*-infected tissues in a mouse model of reactive arthritis

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ABSTRACT

*Chlamydia trachomatis* is an intracellular human pathogen that causes a sexually transmitted disease which may result in an inflammatory arthritis designated *Chlamydia*-induced reactive arthritis (ReA). The arthritis develops after dissemination of infected cells from the initial site of chlamydial infection. During *Chlamydia*-associated ReA, the organism may enter into a persistent infection state making treatment with antibiotics a challenge. We hypothesize that folate receptors (FR), which are overexpressed in *Chlamydia*-infected cells, and the associated inflammation would allow folate-targeted nanodevices to better treat infections. To investigate this, we developed a folate–PAMAM dendrimer–Cy5.5 conjugate (D–FA–Cy5.5), where Cy5.5 is used as the near-IR imaging agent. Uptake of D–FA–Cy5.5 upon systemic administration was assessed and compared to non-folate conjugated controls (D–Cy5.5), using a mouse model of *Chlamydia*-induced ReA, and near-IR imaging. Our results suggested that there was a higher concentration of folate-based nanodevice in sites of infection and inflammation compared to that of the control nanodevice. The folate-conjugated nanodevices localized to infected paws and genital tracts (major sites of inflammation and infection) at 3–4 fold higher concentrations than were dendrimer alone, suggesting that the overexpression of folate receptors in infected and inflamed tissues enables higher dendrimer uptake. There was an increase in uptake into thymus, spleen, and lung, but no significant differences in the uptake of the folate nanodevices in other organs including kidney and heart, indicating the 'relative specificity' of the D–FA–Cy5.5 conjugate nanodevices. These results suggest that folate targeting dendrimers are able to deliver drugs to attenuate infection and associated inflammation in *Chlamydia*-induced ReA.

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1. Introduction

Folate receptor-mediated drug delivery systems have added a new dimension to the therapeutic approach for treatment of cancer and various inflammatory diseases (Sudimack and Lee, 2000; Lu et al., 2004; Hilgenbrink and Low, 2005; Sega and Low, 2008). Under pathological conditions, cells undergo many physical as well as physiological modifications which lead to a deviation from a healthy cell cycle. It is well known that folate receptors (FR) are overexpressed in various human cancer cells but have relatively limited expression in healthy tissues (Leamon and Low, 2001; Leamon and Reddy, 2004). FR are glycosylphosphatidylinositol (GPI)-anchored membrane bound proteins with molecular weight of 38–40 kDa that bind folic acid with high affinity (Shen et al., 1994). The folic acid/FR complex is internalized into the cytoplasm by an endocytic process (Kamen and Capdevila, 1986). A drop in pH inside the endosome leads to the release of folic acid from the complex, and the free FR recycle to the cell surface ready for further binding. The receptor-mediated endocytic process acts in the same manner for folate-conjugated cargos. This mechanism has been exploited for the delivery of small chemotherapeutic drugs (Quintana et al., 2002; Singh et al., 2008; Sulistio et al., 2011), MRI contrast agents (Wiener et al., 1997) and antisense oligonucleotides (Kang et al., 2010; Li et al., 1998).
FR-mediated delivery has an advantage in reducing the uptake of drug by healthy tissue, as more receptors are present in the diseased tissue. Activated mononuclear cells and other inflammatory cells notably have increased numbers of FR compared to resting cells (van der Heijden et al., 2009). Even though this concept has been widely utilized in cancer, applications for targeting inflammatory cells have emerged more recently. Despite a long known ability of *Chlamydia trachomatis* to synthesize folate (e.g., Fan et al., 1992), the upregulation of FR transcription and translation on *Chlamydia*-infected cells, had not been previously described. Chlamydial infections are known to be pro-inflammatory and highly immunogenic, leading to infiltration of infected tissues by activated mononuclear cells and macrophages. Further, recent studies showed upregulation of several FR isosforms on *Chlamydia*-infected cells in vitro and in vivo (Panyam et al., 2010). Therefore, the use of folate-targeted dendrimers may enhance the delivery of therapeutics to sites of *Chlamydia*-infected cells, and is novel.

Reactive arthritis (ReA) is an inflammatory disease which is a subset of spondyloarthropathies. By definition, there is a history of a prior gastrointestinal infection (food poisoning) by *Salmonella, Shigella, or Yersinia* species (Gérard et al., 2008). Alternatively, documented genitourinary tract infections by *C. trachomatis* or *Neisseria gonorrhoeae*, or respiratory infections due to *C. pneumoniae*, also are associated with ReA. Abundant evidence has shown that *Chlamydia*-associated ReA involves the presence of viable organism in synovial tissue (Gérard et al., 2001; Gérard et al., 2010). The other organisms may not be viable, but nonetheless can lead to antigen-driven joint inflammation. Because *Chlamydiae* are viable in joint tissue, better antibiotic therapeutics are desirable. However, the need for antibiotics to cross host cell membranes, the chlamydial inclusion membrane, and finally to cross the chlamydial cell membrane has posed therapeutic challenges. Further, there is abundant evidence that chlamydial infections of ReA represent *Chlamydiae* in an altered state of gene expression termed persistence (e.g., Gérard et al., 2001). Persistent chlamydial infections are particularly difficult to treat in vitro and in vivo (Beutler et al., 1997; Carter et al., 2004; Carter and Inman, 2011). The most successful treatment to date for ReA was reported by Carter et al. (2004) in which systemically delivered combination antibiotics were found to be most successful at clearing organism from patient synovial tissues. If combination drugs could be delivered more efficiently to patients with *Chlamydia*-associated ReA, this would be an important therapeutic advance.

There are many examples of over-expression of FR on macrophages and activated monocytes in various types of arthritis and other inflammatory conditions (Paulos et al., 2004; Vaitilingam et al., 2012). The presence of activated monocyte/macrophages in synovial tissue of arthritic joints plays an active role in the inflammatory process (Nakashima-Matsushita et al., 1999; Turk et al., 2002). It is also established that FR are over-expressed only in activated monocyte/macrophages but not in quiescent cells, both in animal models and in humans (Paulos et al., 2004). There have been studies to develop human monoclonal antibodies specific to FR for the treatment of rheumatoid arthritis and FR positive tumors (Feng et al., 2011). Taking this concept one step further, aminoprin, a precursor of methotrexate was conjugated with folic acid for efficient targeting to sites of inflammation (Lu et al., 2011). To extend the scope of FR-targeted delivery, vehicles such as polyethylene glycol (Yoo and Park, 2004), liposomal nanoparticles (Lu et al., 2007), (hydroxypropyl)-methacrylamide (HPMA) copolymer nanodevices (York et al., 2010; Roger et al., 2012) and polyamidoamine (PAMAM) dendrimers (Majoros et al., 2005) tagged with therapeutics have been used for more efficient and targeted delivery to sites of inflammation.

Dendrimers (size ~3–10nm) are nanostructured delivery vehicles for therapeutic and imaging agents. Dendrimers can be coupled with siRNA, short DNA, plasmid DNA and oligonucleotides for different biomedical applications due to dendrimer small globular size, branching architecture, and high density tailorable surface functional groups (Lee et al., 2005; Svenson and Tomalia, 2005). PAMAM dendrimers are the most widely studied class due to their commercial availability, with a large number of animal studies and pre-clinical studies currently underway in cancer therapy, imaging, and targeted delivery applications (Menjoje et al., 2010; Navath et al., 2010; Han et al., 2010). Recent studies have explored the use of dendrimers for the treatment of systemic inflammation (Bosnjakovic et al., 2011; Chauhan et al., 2009), and neuro-inflammation (Dai et al., 2010; Kannan et al., 2012). The delivery of indomethacin, a non-steroidal anti-inflammatory drug to the site of inflammation in a rodent arthritis model has been reported using a folate-dendrimer platform (Chandrasekar et al., 2007). More recently, methotrexate, an anti-folate drug has been conjugated to a dendritic platform of folate for receptor-based delivery in the collagen-induced arthritis rat model (Thomas et al., 2011). Azabis-phosphonate (ABP)-capped dendrimers have been used to reduce the inflammatory and bone-erosing effects in a rheumatoid arthritis (RA) mouse model (Hayder et al., 2011). These studies suggested that folate-mediated dendritic vehicles can target activated monocytes in the synovial tissue of a ReA animal model.

It was reported previously that dendrimers can rapidly enter *Chlamydia*-infected cells in vitro and concentrate in chlamydial inclusions (Mishra et al., 2011). We now report that a physiological murine model of *Chlamydia*-associated ReA allows testing of folate-targeted delivery of dendrimers to infected and infected tissues in murine ReA. Murine ReA develops following a chlamydial ocular or genital infection in which organism disseminates to knee joints and paws within 7 days after initial mucosal infection and local inflammation develops (Whittum-Hudson et al., 1999). This model recapitulates what is observed in clinical ReA which develops after urogenital *C. trachomatis* infections (Gérard et al., 2010). The present study focused on whether dendrimer–folate conjugates would localize to the infected and inflamed synovial tissue of this ReA, with a view to develop targeted therapies for ReA using antibiotics such as Azithromycin (Mishra et al., 2011). Cy5.5-labeled folate-conjugated dendrimers (D–FA–Cy5.5) were developed and imaged in vivo in our murine model to test FA-enhanced targeting of dendrimers to sites of infection and inflammation. The FR-targeted nanodevices were taken up into inflamed, infected paws and genital tracts (major sites of inflammation and infection) at significantly higher levels than dendrimer alone and supported successful folate-dendrimer targeting of inflamed and/or *Chlamydia*-infected tissues.

2. Materials and methods

2.1. Materials

Generation four amine-terminated PAMAM dendrimers (“D”) (MW = 14,200 Da) were purchased from Dendritech Inc. (Midland, MI, USA). Folic acid (FA), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (EDC) and N-hydroxysuccinimidiozole (HOBT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cy5.5 mono NHS ester was purchased from Amersham Biosciences (GE Healthcare). Deuterated water (D2O) and dimethylsulfoxide (DMSO–d6) were purchased from Cambridge Isotope Laboratories. Triethylamine (TEA) and diisopropyl-ethylamine (DIEA) were purchased from Thermo Fisher Scientific (St Louis, MO, USA). Trifluoroacetic acid (TFA), dimethyformamide (DMF) and dimethylsulfoxide (DMSO) were purchased from EMD Chemicals (Billerica, MA, USA). All other solvents and chemicals were purchased from ThermoFisher and used without further purification. Dialysis membrane (Molecular weight cut-off of 1000 Da) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA).
2.2. Proton NMR characterization

Proton NMR spectra were recorded on a Varian INOVA (400 MHz) spectrometer using commercially available deuterated solvents. Proton chemical shifts were reported in ppm (δ) and tetramethylsilane (TMS) used as an internal standard. Coupling constants (J) were reported in hertz (Hz).

2.3. High performance liquid chromatography

The purity of the conjugates was evaluated with a Waters HPLC instrument. The HPLC system consisting of an auto sampler (Waters 717) equipped with 150 μL loop, a dual UV detector (Waters 2487) and two pumps interfaced with breeze software. A C18 silica based reverse phase-HPLC column (250 × 5 mm, 300 Å) equipped with two C5 Supelguard cartridges (2 cm × 4 mm, 5 μm) was used for this study. A freshly prepared, filtered and degassed water/acetonitrile mixture (0.14% TFA) was used as mobile phase prior to the use. A linear gradient of acetonitrile/water (10:90) to 32:68 in 25 min and back to initial concentration in 40 min at a flow rate of 1 ml/min was used. 50 μL of each sample was injected and detection was performed at 210 nm (G4-NH2), 288 nm (folic acid) and 675 nm for D-Cy5.5 and D-FA-Cy5.5.

2.4. MALDI-TOF mass characterization

MALDI-TOF mass spectra were recorded on a Bruker Ultraflex system (Bruker Corporation, Billerica, MA, USA) equipped with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and 2.5-dihydroxybenzoic acid (DHB) was used as matrix. The conjugates were dissolved in DMF/DMSO (1:1, 10 mg/ml) and the matrix 2.5-dihydroxybenzoic acid was dissolved in acetonitrile/water (1:1, 20 mg/ml). The dissolved conjugate (2 μl) was mixed with 20 μl of DHB solution and 1 μl of the sample was spotted on a Bruker Daltons MALDI plate. The sample was further laser irradiated in a Bruker MALDI instrument.

2.5. Synthesis of dendrimer–Cy5.5 (D–Cy5.5, 1)

To a stirred solution of the dendrimer (30 mg, 0.0021 mmol) in NaHCO3 buffer solution (pH 8.5, 3 ml), Cy5.5 NHS ester (2.4 mg, 0.0021 mmol) dissolved in DMSO (2 ml) was added under nitrogen atmosphere, and the reaction mixture was stirred overnight in the dark at room temperature. The solution was freeze-dried and reconstituted with water and purified by dialysis (membrane MW cutoff 1000 Da) against DI water. The resulting solution was lyophilized to obtain dendrimer–Cy5.5 (D–Cy5.5, 1) and characterized by reverse phase-HPLC and MALDI-TOF mass.

2.6. Synthesis of dendrimer–folic acid (D–FA, 2)

Folic acid (308 mg, 0.7 mmol) was dissolved in DMSO/NaHCO3 (1:1) buffer solution (20 ml) and EDC (201 mg, 1.05 mmol), N-hydroxybenzotriazole (141 mg, 1.05 mmol) and triethylamine (141 mg, 1.40 mmol) were added. The resulting reaction mixture was stirred for 1 h under nitrogen, and generation four PAMAM dendrimer (250 mg, 0.0175 mmol) dissolved in DMSO (15 ml) was added and the reaction mixture was stirred for 48 h. The resultant solution was freeze-dried and reconstituted with water and purified by dialysis (MW cutoff 1000 Da) against DI water for 24 h and lyophilized. The lyophilized product was further purified by gel permeable chromatography (GPC) using Sepharose 4B against DI water and again lyophilized to get dendrimer–folic acid conjugate (D–FA, 2). The conjugate was characterized by proton NMR and reverse phase HPLC. 1H NMR (D2O) δ 1.65–1.72 (m, 2H, —CH2 of FA), 1.88–2.04 (m, 2H, —CH2 of FA), 2.13–2.30 (m, —CH2 of G4-OH), 2.40 (bs, —CH2 of G4-OH), 2.81–3.22 (m, —CH2 of G4-OH), 4.13–4.17 (m, 1H, —CH protons of FA), 4.40 (bs, 2H, —CH2 of FA), 6.63–6.65 (d, 2H, aromatic protons of FA), 7.24–7.28 (m, 2H, NH2 of FA), 7.52–7.54 (d, 2H, aromatic protons of FA), 7.65–7.67 (d, 1H, NHCO of FA), 8.45 (s, 1H, CH = N of FA).

2.7. Synthesis of dendrimer–folic acid–Cy5.5 (D–FA–Cy5.5, 3)

Dendrimer–folic acid conjugate, D–FA (36 mg, 0.0016 mmol) was dissolved in DMSO/NaHCO3 (1:1) buffer solution (5 ml) and Cy5.5 NHS ester (1.8 mg, 0.0016 mmol) dissolved in DMSO (2 ml) was added to it under nitrogen atmosphere and the reaction mixture was stirred overnight in dark at room temperature. The resulting reaction mixture was lyophilized and reconstituted with water and dialyzed against DI water (MW cutoff 1000 Da) for 24 h. The resulting solution was lyophilized to obtain dendrimer–folic acid–Cy5.5 conjugate (D–FA–Cy5.5, 3). The final conjugate was characterized by reverse phase HPLC and MALDI-TOF mass.

2.8. Chlamydia trachomatis infection of mice

Female BALB/c mice (5–6 weeks old) were obtained from Charles River Breeders (Wilmington, MA, USA). Mice were treated with DepoProvera (2.5 mg/mouse; Pharmacia & Upjohn, Kalamazoo, MI, USA) seven days prior to chlamydial infection. Chlamydia trachomatis (K serovar, UW-31) stocks were prepared using our published methods (Whittum-Hudson et al., 2001). Briefly, HEp2 monolayers were infected with a multiplicity of infection (MOI) of approximately 5 and grown for 72 h in infection medium (Isocve’s modified Dulbecco’s medium (GIBCO, Grand Island, NY, USA)) supplemented with 10% heat-inactivated fetal bovine serum (Atlantic Biologicals, Lawrenceville, GA, USA), 2 mM l-glutamine (Sigma–Aldrich, St. Louis, MO, USA), 0.0594% glucose (Sigma–Aldrich), and 0.2 μg/ml cycloheximide (Sigma–Aldrich). Per-coll purified elementary bodies were titrated on McCoy cells. Aliquots of Chlamydia were stored at ~80 °C until use.

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg; Ketaset, Fort Dodge, IO) and xylazine (8 mg/kg; Anased, Shenandoah, IO, USA). Mice were infected by topical vaginal delivery of 4000 TCID50 C. trachomatis in 30 μl sucrose-phosphate-glutamate (SPG). Mice were left on their backs until recovery from anesthesia (~30 min) to improve in vivo adsorption and uptake of bacteria. To maximize the infection and inflammatory response, the mice used for this study received a second infection of 2000 TCID50 five weeks after the initial genital infection. One week after reinfection, mice were randomized to three groups of three mice each. Each mouse received dendrimer-conjugates by the intravenous (tail vein) route. One group of infected mice received DFA/Cy5.5 (172 μg/mouse). A second group of infected mice received D–Cy5.5 without folate (120 μg/mouse). A third group of uninfected, control mice also received D–FA–Cy5.5 (172 μg/mouse, containing equivalent amount of dendrimer as in the D–Cy5.5 group). Doses were normalized so that all mice received the same amount of Cy5.5. Mice were handled under an approved Wayne State University IACUC protocol (A11-09-09).

2.9. Whole animal imaging of D–Cy5.5 localization

All mice were imaged with a whole body animal imager (In Vivo-Kodak Image Station IS4000MM with X-Ray, Carestream Health, Carestream Molecular Imaging, Woodbridge, CT, USA) at 2 h, 24 h, 48 h, and 120 h after dendrimer conjugate injections. For imaging, mice were anesthetized with Isoflurane (Butler Schein Animal Health, Inc., Fort Worth, TX, USA) at 4% induction and at 2% for maintenance during imaging. Whole body images were
collected at the same settings for all mice using software provided with the instrument (Carestream Molecular Imaging Software (CMI)). Imaging was done at the following wavelengths: excitation at 625 nm, emission at 700 nm for 30 s of exposure. After the final images were collected, mice were euthanized for tissue collection. Front and back paws, hind knees, genital tract, liver, kidney, spleen, thymus and hearts were collected for ex vivo imaging.

2.10. Image analysis of Cy5.5 localization

Collected images were imported as TIFF files into ImagePro Plus v.6.3.0.512 (Media Cybernetics, Silver Spring, MD) for analysis of Cy5.5 localization and intensity, based on mean pixel density. Pixel densities of Cy5.5 fluorescence were determined for whole animals (paws) and for ex vivo tissues (front and hind paws, genital tract, thymus, heart, lung, liver and kidneys) by delineation of areas of interest (AOI). The mean pixel densities for tissues from each treatment group were compared. Significance of differences based on treatment was determined using SigmaPlot (v.11.2; Systat, Inc. San Jose, CA, USA).

3. Results and discussion

3.1. Preparation of dendrimer–Cy5.5 (D–Cy5.5, 1)

We previously reported the conjugation of hydroxy-terminated PAMAM dendrimers with the near IR imaging probe Cy5.5. The conjugate was prepared by reacting amine-functionalized bifunctional dendrimer with Cy5.5 mono-NHS ester to learn the biodistribution of dendrimer in retina (Iezzi et al., 2012). We preferred Cy5.5 as the imaging probe since it shows relatively low auto-fluorescence in the near IR region (Rao et al., 2007). In the present study, we developed a Cy5.5-conjugated, amine-terminated generation four PAMAM dendrimer for the biodistribution study in the chlamydial infection model. Cy5.5 mono-NHS ester was reacted with the dendrimer in sodium bicarbonate buffer solution at room temperature overnight, as shown in Fig. 1(A). D–Cy5.5 was purified by dialysis and freeze-dried (D–Cy5.5, 1). A stable amide linkage was established between Cy5.5 and dendrimer; formation of D–Cy5.5 was characterized by reverse phase-HPLC and MALDI-TOF mass. In the HPLC trace, a single peak at 11.4 min at 675 nm (λ_max of Cy5.5) for D–Cy5.5 which is different from dendrimer (9.8 min, 210 nm) and free Cy5.5 (monitored at 675 nm) confirmed the formation of the product (Fig. S1). In the same retention time, we did not see any peak at 210 nm, further confirming that no free dendrimer was present. A molecular weight peak at 15,060 Da in MALDI-TOF mass spectrum of D–Cy5.5 suggested that only one molecule was attached to the dendrimer, considering that the molecular weight of the dendrimer is 13,980 Da. In the reaction, we used 1 mole equivalent of Cy5.5 per dendrimer molecule, and we show in MALDI-TOF mass data that 1 molecule was attached, suggesting an efficient reaction. The Cy5.5 payload in the dendrimer was estimated to be 6–7%, and the conjugate was stable in PBS buffer over a period of one week.

3.2. Preparation of dendrimer–folic acid–Cy5.5 (D–FA–Cy5.5, 3)

A folate receptor-targeting nanodevice was developed by reacting folic acid and Cy5.5 with dendrimer to compare its biodistribution with a nanodevice having no targeting ligand in an animal model of Chlamydia-associated ReA (Whittum-Hudson et al., 1999). The preparation of the D–FA–Cy5.5 involved a two-step synthetic protocol. First, folic acid (FA) was reacted with the PAMAM dendrimer under basic conditions using EDC as coupling agent,
to get dendrimer–FA (D–FA, 2) as shown in Fig. 1(B) (Shukla et al., 2003; Kono et al., 1999; Cheng et al., 2006). The resulting conjugate has a stable amide bond between dendrimer and FA and was characterized by reverse phase HPLC and 1H NMR spectroscopy. In the HPLC chromatogram, a broad peak at 10.0 min (monitored at 288 nm), which is different from dendrimer (9.8 min, 210 nm) and FA (9.7 min, 288 nm), suggested the formation of the desired conjugate. A comparatively less broad peak of D–FA is due to the low polydispersity of the conjugate and the conjugate is pure since there is no measurable amount of folic acid present as shown in HPLC chart (Fig. S2). The 1H NMR spectrum showed two doublets at 6.64 and 7.53 ppm representing the aromatic protons of FA; three multiplets in between 1.65 and 2.04 ppm representing aliphatic protons of FA along with interior aliphatic protons peaks of the dendrimer from 2.00 to 3.22 ppm, confirmed the formation of the conjugate, D–FA (Fig. S3 for FA and Fig. S4 for D–FA). The loading of FA to the dendrimer was estimated by proton NMR, by comparing proton integration of the interior aliphatic protons of the dendrimer and aromatic protons of FA. We estimated that 16 molecules of FA were reacted per dendrimer molecule. The intermediate D–FA conjugate was soluble in water as well as in PBS buffer unlike free FA. Finally, Cy5.5 NHS-mono ester was reacted with the D–FA conjugate under basic condition, yielding a FR targeting nanodevice (D–FA–Cy5.5, 3, Fig. 1(B)). This conjugate was characterized by reverse phase HPLC (for purity) and MALDI-TOF mass (for molecular weight). In the HPLC chromatogram, a peak at 10.6 min (monitored at 675 nm), which is different from D–FA (10.0 min, 288 nm), supported formation of the desired conjugate with no free D–FA and Cy5.5 present. A peak at 22.310 in MALDI-TOF mass spectrum showed that only one molecule of Cy5.5 is conjugated to the dendrimer given the molecular weight of D–FA (21,360 Da).

3.3. Targeted in vivo localization of dendrimer–folic acid conjugates to areas of infection and inflammation

Infected and uninfected mice were imaged in the whole animal Kodak imager at 2, 24, 48 and 72 h after intravenous delivery of dendrimer conjugates. The settings during imaging were kept constant for all mice at all time points. Mice were coded so that image analyses were performed without knowledge of treatments. For in vivo imaging of whole animals, only paws showed detectable localized fluorescence as shown in images collected at 2 h after injections (Fig. 2). The imager was not capable of detecting internally localized fluorescence. Raw TIFF images were collected from the animal imager and analyzed with ImagePro Plus, as described. The values for each set of four paws/mouse were averaged and expressed as mean pixel density ± standard error. FA significantly enhanced dendrimer targeting to paws of infected mice at all time points of imaging. Dendrimer-conjugates rapidly reached paws harboring Chlamydia and inflammatory cells: even at 2 h after injection of dendrimer conjugates, infected recipients of D–FA–Cy5.5 exhibited significantly higher pixel densities compared to control mice (Fig. 3(A)). The concentration of Cy5.5 signal was indicative of dendrimer localization in paws and tissues (see Section 3.3.2), because of the covalent linkage between Cy5.5 and dendrimers (relatively minimal Cy5.5 release from the conjugate during this time) (Lesniak et al., 2013). Free Cy5.5 did not concentrate in any tissues based on in vivo and ex vivo animal imaging of a subset of mice (not shown).

3.3.1. In vivo imaging

In vivo fluorescence could be detected in all animals up to 120 h after dendrimers were administered intravenously (Fig. 3(A–C)).
The \textit{in vivo} fluorescence was the highest in \textit{Chlamydia}-infected recipients of D–FA–Cy5.5, which persisted at \textasciitilde{}150 pixel units from the initial time point of 2 h, up to the final time point of 120 h (Fig. 3A–C). At all time points, the mean pixel densities were higher (by more than 3–4-fold) in D–FA–Cy5.5 paws, compared to uninfected D–Cy5.5 in infected animals. The differences in the uptake were even more significant between D–FA–Cy5.5 uptake in infected and uninfected animals, with the uptake being at least 10-fold higher in infected paws ($p < 0.001$). The concentration of Cy5.5 in the paws and genital tracts suggests that the folate targeting of dendrimers is effective in targeting inflammatory cells as well as infected cells. Such targeted concentration would be important for \textit{in vivo} delivery of therapeutics and anti-inflammatory agents.

### 3.3.2. Ex \textit{vivo} imaging

All animals were euthanized at 120 h after nanodevice delivery, and the \textit{ex vivo} fluorescence in dissected paws and genital tracts (two key areas affected by the chlamydial infection) was analyzed. Additional tissues were removed for \textit{ex vivo} imaging to support specificity of targeted dendrimers: spleen, thymus, liver, kidneys, lung, and heart. As expected from the \textit{in vivo} imaging results, paws from the infected animals that received the targeted nanodevice, D–FA–Cy5.5, showed significantly higher fluorescence compared to that observed for the same compound in uninfected animals. Importantly, the fluorescence signal detected after delivery of D–FA–Cy5.5 also greatly surpassed that detected with the non-targeted dendrimer (D–Cy5.5) in infected animals ($p < 0.001$; Fig. 4). The genital tract (GT), thymus, spleen, and lung from infected mice which received D–FA–Cy5.5 exhibited significantly enhanced pixel densities compared to the same tissues from infected mice who received the D–Cy5.5 (non-targeted) and uninfected mice who received D–FA–Cy5.5. The uptake of targeted nanodevice in the tissues from infected mice was 3–4-fold higher than the uptake of non-targeted dendrimer ($p < 0.004$–0.001). The results of \textit{ex vivo} imaging were internally consistent with the \textit{in vivo} imaging results, and further support the potential of FR-targeted dendrimer delivery of therapeutics in chlamydial infections. To our knowledge, no information regarding FR expression in cells infected with other intracellular pathogens (bacterial or viral) is available but would warrant future study. Importantly, if FR upregulation is a global phenomenon in such infections, the use of dendrimer–antibiotic conjugates would have broader medical therapeutic application.

The increased D–FA–Cy5.5 localization in GT is believed to be due to the up-regulation of FR in that tissue both because of presence of activated monocytes and other inflammatory cells as well as infected GT epithelial cells, all of which have higher levels of FR (Xia et al., 2009). Chlamydial infections are pro-inflammatory and result in inflammatory cell migration to the genital tract and synovial tissue. Further, we have observed that \textit{Chlamydia}-infected cells upregulate several FR isoforms which would enhance folate targeting (Panyam et al., 2014) (Fig. 4). Mouse thymus and spleen have been reported to express FR Folbp1, 2 and 3, and lung tissue expresses traces levels of the latter (Spiegelstein et al., 2000). Folbp1 and 2 have homology to human FRα and β, respectively. Spiegelstein et al. (2000) proposed that Folbp3 is the ortholog of human FRγ. Folbp is highly expressed in mouse thymus and spleen and presumably resulted in D–FA targeting to those tissues; enhanced localization in lung of infected mice by D–FA correlates with the latter studies of Spiegelstein et al. Circulation of activated monocytes and lymphocytes after chlamydial infection could result in increased FA receptor expression in spleen cells to further explain the significant localization of D–FA in this organ. There were no significant differences in localization of dendrimers in hearts, liver or kidneys in infected and uninfected mice regardless of nanodevice conjugation.

\begin{figure}[h]
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\caption{\textit{Ex vivo} imaging of paws and organs for folate receptor-targeted Cy5.5-dendrimers (D–FA–Cy5.5). Paws and tissues were removed from each mouse after the 120 h \textit{in vivo} whole animal imaging. Specific tissues were removed and all imaged at the same time (e.g., all genital tracts) by placement on sterile petri dishes; image collections were done at the same time interval (30 s). Images from all tissues were analyzed with ImagePro software under the same settings. Localization of fluorescent dendrimers was expressed as mean pixel density ± SE for areas of interest. Treatments were compared by One Way Analysis of Variance with pairwise comparisons done by the Student–Newman–Keuls method; * $p < 0.004$, ** $p < 0.001$. Significantly more D–FA localized to tissues of infected mice compared to untargeted D; no significant differences were observed between the different treatment groups for heart, kidney and liver. D–FA: folate-targeted dendrimer labeled with Cy5.5; and D: only the dendrimer labeled with Cy5.5.}
\end{figure}
4. Conclusions

We successfully targeted FA-conjugated dendrimers to inflamed and Chlamydia-infected mouse tissues, particularly paws and GTs. The paws and GTs from infected mice exhibited 4–5 fold higher pixel densities of D–Cy5.5 when conjugated with FA compared to the D–Cy5.5 without FA. While we know that activated mononuclear cells and lymphocytes as well as Chlamydia-infected mononuclear cells localize in synovial tissue of knees of this model (Whittum-Hudson et al., 1999), the numbers appeared to be below detection levels of the Kodak imager, which best detects Cy5.5 signal near skin surfaces. In addition, D–Cy5.5 localization to the synovial membrane may have been obscured by bone. Uninfected mice had no significant localization of D–FA–Cy5.5 conjugate in any tissue based on pixel densities from Cy5.5. We know that activated macrophages and monocytes up-regulate FR allowing dendrimers to concentrate at sites of inflammation accompanying chlamydial infection. Our results are consistent with the synergistic effect of Chlamydia-infected cells with up-regulated FR at sites of disseminated infection coupled with the inflammatory cell infiltration. Perhaps we can use the potential conjugation property of dendrimers to allow delivery and release of antibiotics in sites of infection and associated inflammation to eradicate Chlamydia from these tissues. This ability would offer new therapeutic approaches with dendrimer–antibiotic conjugates for chronic pro-inflammatory infections such as Chlamydia-associated ReA. Application to other intracellular pathogens including other bacteria and viruses could provide a future generation of targeted dendrimer-therapeutics for infectious diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ajca.2013.12.001.

References


