

Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation

Sunil Shaunak¹, Sharyn Thomas¹, Elisabetta Gianasi¹, Antony Godwin², Emma Jones³, Ian Teo¹, Kamiar Mireskandari³, Philip Luthert⁴, Ruth Duncan⁵, Steve Patterson⁶, Peng Khaw³ & Steve Brocchini²

Dendrimers are hyperbranched macromolecules that can be chemically synthesized to have precise structural characteristics. We used anionic, polyamidoamine, generation 3.5 dendrimers to make novel water-soluble conjugates of D(+)-glucosamine and D(+)-glucosamine 6-sulfate with immuno-modulatory and antiangiogenic properties respectively. Dendrimer glucosamine inhibited Toll-like receptor 4-mediated lipopolysaccharide induced synthesis of pro-inflammatory chemokines (MIP-1 α , MIP-1 β , IL-8) and cytokines (TNF- α , IL-1 β , IL-6) from human dendritic cells and macrophages but allowed upregulation of the costimulatory molecules CD25, CD80, CD83 and CD86. Dendrimer glucosamine 6-sulfate blocked fibroblast growth factor-2 mediated endothelial cell proliferation and neoangiogenesis in human Matrigel and placental angiogenesis assays. When dendrimer glucosamine and dendrimer glucosamine 6-sulfate were used together in a validated and clinically relevant rabbit model of scar tissue formation after glaucoma filtration surgery, they increased the long-term success of the surgery from 30% to 80% ($P = 0.029$). We conclude that synthetically engineered macromolecules such as the dendrimers described here can be tailored to have defined immuno-modulatory and antiangiogenic properties, and they can be used synergistically to prevent scar tissue formation.

Dendrimers are hyperbranched synthetic macromolecules that can be made using controlled sequential processes to give them defined structural and molecular weight characteristics. This endows them with a structural precision that is similar to low molecular weight molecules^{1,2}. Dendrimers have been evaluated for biomedical applications that include drug delivery and the conjugation of pharmacologically active compounds³.

There is increasing recognition of the importance of polyvalent receptor-ligand interactions between carbohydrates and proteins in many aspects of cell surface-mediated immuno-regulation^{4,5}. Therefore, any new medicine that aims to effectively modulate these interactions must possess multiple and cooperative receptor binding properties. Previous attempts to pharmacologically manipulate these interactions with synthetic linear polymers were impaired by the structural heterogeneity of the polymers and by their ability to activate complement and coagulation pathways^{6,7}.

The chemical functionality of dendrimer end groups can be modified to make molecules with novel biological properties that exploit polyvalent and cooperative receptor-ligand interactions^{8,9}. Whereas dendrimers with cationic end groups are often toxic after repeated intravenous use or topical ocular application^{10,11}, anionic dendrimers are not¹². This led us to postulate that anionic dendrimer conjugates of aminosaccharides and sulfated aminosaccharides

could be made via manipulation of their end groups to have defined immuno-modulatory and antiangiogenic properties, respectively. In this study, we describe two optimized conjugates that were safely used in combination to synergistically prevent scar tissue formation after surgery.

RESULTS

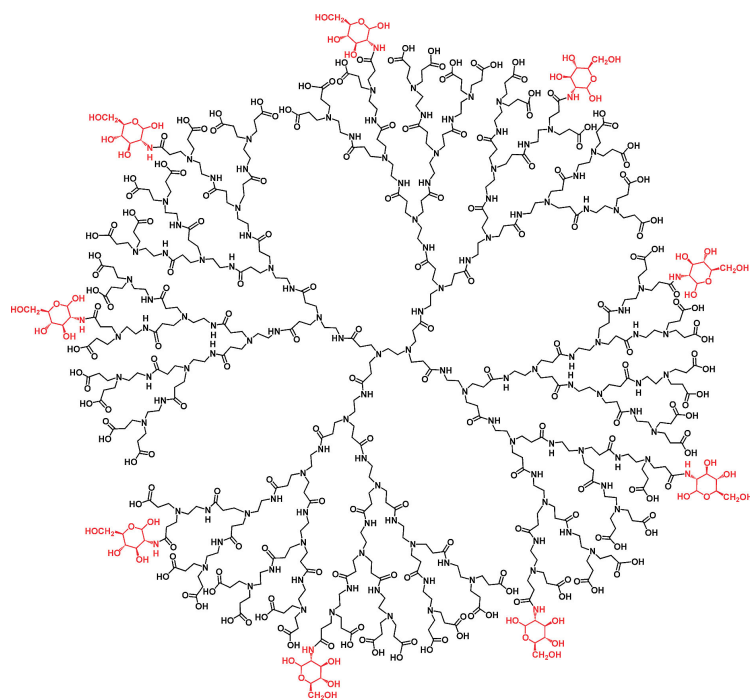
Synthesis of glycoconjugates

We conjugated the aminosaccharides glucosamine and glucosamine 6-sulfate to a generation 3.5 polyamidoamine (PAMAM) dendrimer (Dendritech) that had 64 carboxylic acid end groups. These two dendrimer glycoconjugates, dendrimer glucosamine (Fig. 1a) and dendrimer glucosamine 6-sulfate, were prepared with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) at pH 5 to minimize *N*-acyl isourea formation. The total aminosaccharide conjugation was estimated by ¹H-NMR in D₂O (Fig. 1b,c). The integral for both anomeric forms of the saccharide C-1 proton (C-1 H α and H β signals at 5.2 p.p.m. and 4.8 p.p.m. respectively for both dendrimer glucosamine and dendrimer glucosamine 6-sulfate) was used as the diagnostic signal for the covalently bound saccharide. From the integrals of the remaining signals (2.4 to 4.0 p.p.m. for dendrimer glucosamine and 2.4 to 4.4 p.p.m. for dendrimer glucosamine 6-sulfate), we deduced the integral for one proton of

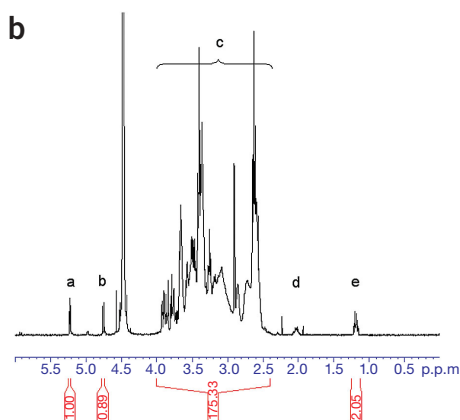
¹Faculty of Medicine, Imperial College London, Hammersmith Hospital, Duane Road, London, W12 0NN, UK. ²Biomedical Polymers Group, School of Pharmacy, 29-39 Brunswick Square, London, WC1N 1AX, UK. ³Wound Healing Research Unit, Institute of Ophthalmology, 11-43 Bath Street, London, EC1V 9EL, UK.

⁴Department of Histopathology, Institute of Ophthalmology, 11-43 Bath Street, London, EC1V 9EL, UK. ⁵Center for Polymer Therapeutics, Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3XF, UK. ⁶Faculty of Medicine, Imperial College London, Chelsea & Westminster Hospital, 369 Fulham Road, London, SW10 9NH, UK. Correspondence should be addressed to S.S. (s.shaunak@imperial.ac.uk).

a



b



c

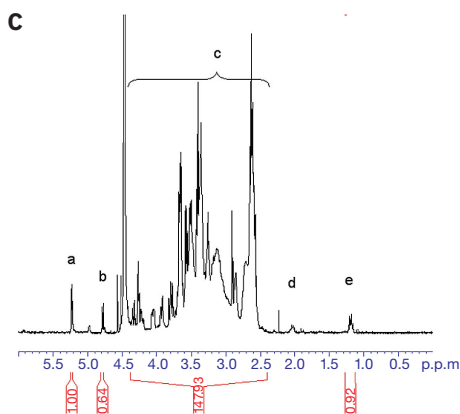


Figure 1 Polyvalent dendrimer glucosamine conjugates. (a) Representative structure of protonated, generation 3.5 PAMAM dendrimer glucosamine. (b,c) ^1H NMR spectra (D_2O) for dendrimer glucosamine (b) and dendrimer glucosamine 6-sulfate (c). a, C-1 H_α aminosaccharide; b, C-1 H_β aminosaccharide; c, 6H aminosaccharide + 12H urea + 740H dendrimer; d, 2H urea, $-\text{CH}_2\text{CH}_2\text{CH}_2-$; e, 3H urea, CH_3CH_2- .

dendrimer (Supplementary Notes online). We used the ratio of aminosaccharide to dendrimer to determine the conjugation loading and expressed it as a percentage of the 64 carboxylic acid groups. The percentage loading of glucosamine and glucosamine 6-sulfate on the dendrimer was 14%. A total of 20 batches each of dendrimer glucosamine (MW = 13.6 kDa) and dendrimer glucosamine 6-sulfate (MW = 14.0 kDa) were made. Each batch was characterized before pooling them for detailed biological evaluation. The conjugates were stable after 12 month storage at 4°C .

Cytotoxicity of glycoconjugates

The 50% lethal dose (LD_{50}) of dendrimer glucosamine for the T cell line, SupT1, was $1,825\text{ }\mu\text{g/ml}$ ($134\text{ }\mu\text{M}$) $\pm 227\text{ }\mu\text{g/ml}$ and for the macrophage cell line U937, $2,837\text{ }\mu\text{g/ml}$ ($209\text{ }\mu\text{M}$) $\pm 107\text{ }\mu\text{g/ml}$. The LD_{50} of dendrimer glucosamine 6-sulfate for SupT1 cells was $307\text{ }\mu\text{g/ml}$ ($22\text{ }\mu\text{M}$) $\pm 29\text{ }\mu\text{g/ml}$ and for U937 cells, $271\text{ }\mu\text{g/ml}$ ($19\text{ }\mu\text{M}$) $\pm 19\text{ }\mu\text{g/ml}$. When peripheral blood mononuclear cells (PBMCs), monocyte-derived macrophages (MDMs), immature monocyte-derived dendritic cells (DCs), $\text{CD}3^+$ T lymphocytes and human umbilical vein endothelial cells (HUVECs) were cultured with dendrimer glucosamine ($200\text{ }\mu\text{g/ml}$) or dendrimer glucosamine 6-sulfate ($100\text{ }\mu\text{g/ml}$) for 5 d, we saw no adverse effect on cell viability or growth. Unlike other sulfated macromolecules, dendrimer glucosamine 6-sulfate had no anticoagulant, heparin-like or complement-activating activity when it was added to fresh whole blood^{6,13}.

Inhibition of LPS cytokine release by dendrimer glucosamine

Preliminary experiments confirmed that dendrimer glucosamine and dendrimer glucosamine 6-sulfate did not alter the spontaneous release of chemokines (that is, MIP-1 α and MIP-1 β) or cytokines (that is, TNF- α and IL-1 β) from resting PBMCs, MDMs or DCs. We then stimulated these cells with lipopolysaccharide (LPS; range 0.05–100 ng/ml) and found that 10 ng/ml caused the maximum release of MIP-1 β and TNF- α into culture supernatants over a period of 21 h without inducing cellular toxicity¹⁴. *Salmonella minnesota* LPS was used because it acts through Toll-like receptor (TLR) 4 on macrophages and DCs, and because cDNA microarray studies of human monocytes and DCs have defined its sequential pattern of pro-inflammatory chemokine followed by cytokine release^{15,16}.

Single donor PBMCs were then cultured with dendrimer glucosamine for 30 min followed by LPS for 21 h. There was a large and significant reduction in the release of the pro-inflammatory chemokines macrophage inflammatory protein (MIP)-1 α (CCL3), MIP-1 β (CCL4) and interleukin (IL)-8 (CXCL8) (Fig. 2a–c), and the pro-inflammatory cytokines tumor necrosis factor (TNF)- α , IL-1 β and IL-6 (Fig. 2d–f). When we repeated this experiment by first exposing PBMCs to LPS and then adding dendrimer glucosamine 2 h or 4 h later, a significant inhibition of pro-inflammatory mediator release was still seen; the only exception was TNF- α when dendrimer glucosamine was added 4 h after the LPS (Fig. 2d). The 50%

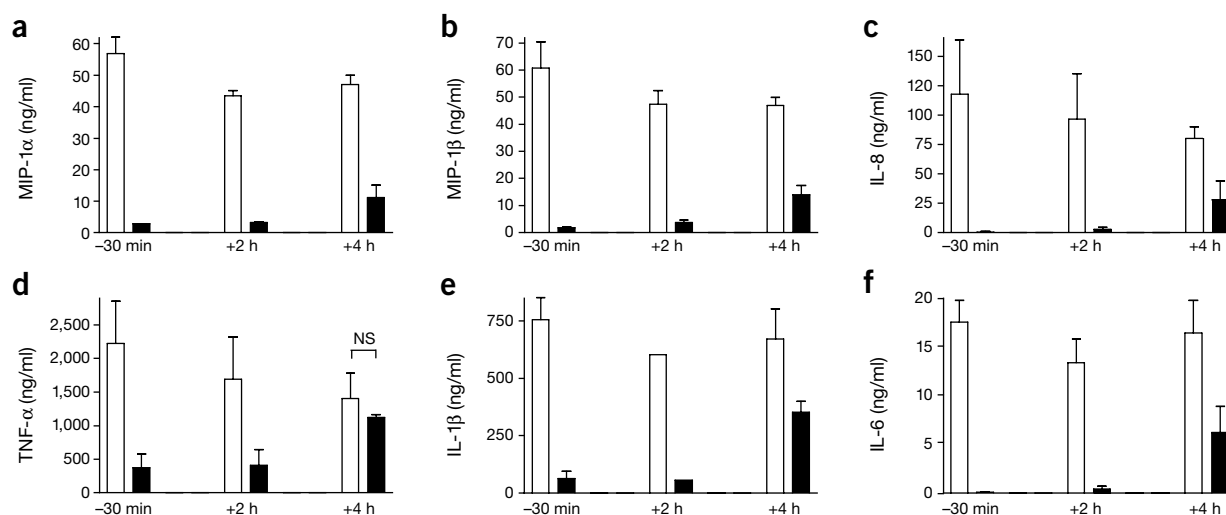


Figure 2 Inhibitory effect of dendrimer glucosamine on LPS-mediated chemokine and cytokine release (**a–f**) Dendrimer glucosamine-inhibited pro-inflammatory chemokine (MIP-1 α , MIP-1 β , IL-8) (**a–c**) and cytokine (TNF- α , IL-1 β , IL-6) (**d–f**) release from human PBMCs when added 30 min before, or 2 h or 4 h after LPS. $n = 3$; $P < 0.001$. White bars, PBMCs + LPS; black bars, PBMCs + dendrimer glucosamine + LPS. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; DG, dendrimer glucosamine; NS, not significant $P > 0.05$.

inhibitory concentration (IC_{50}) of dendrimer glucosamine was $92 (6.8 \mu M) \pm 15 \mu g/ml$; ($n = 4$) (Fig. 3a,b). In further experiments, we used purified populations of cells to demonstrate that the primary effect of dendrimer glucosamine was on DCs and MDMs (Fig. 3c,d and Table 1), and that its immuno-modulatory activity was reversible (Fig. 3e,f). Neither the dendrimer intermediate formed in

the presence of the coupling agent only, nor glucosamine, nor the dendrimer had biological activity.

Inhibition of DC-mediated functions by dendrimer glucosamine

We then examined the ability of dendrimer glucosamine to interfere with DC maturation, activation and function. Treatment with

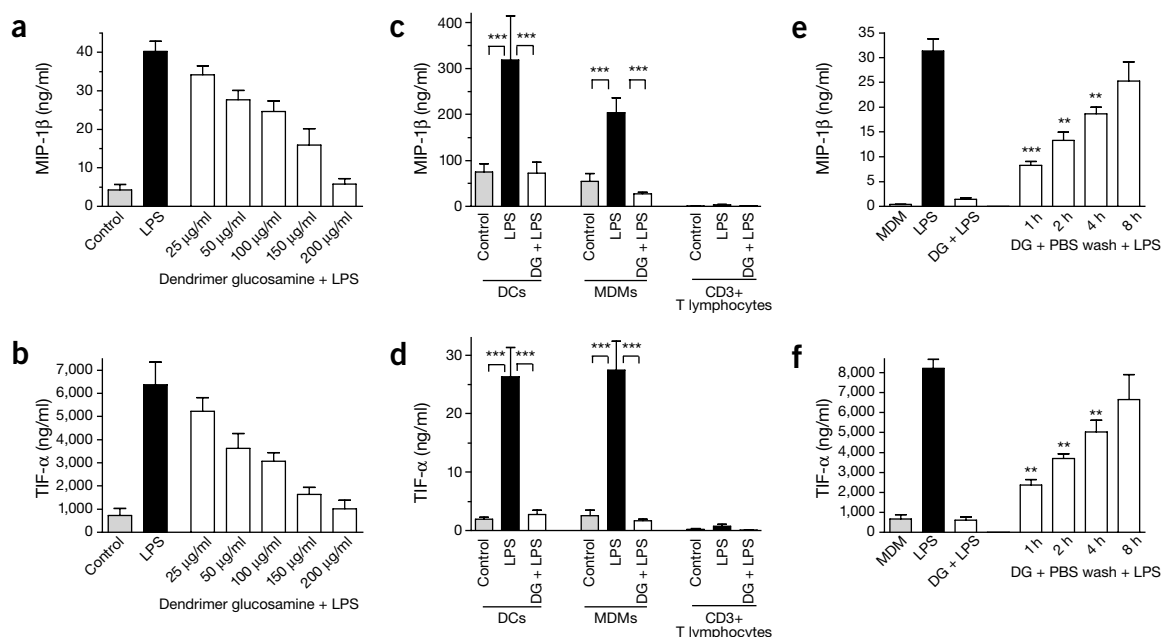


Figure 3 Cell type specific effect of dendrimer glucosamine. (**a,b**) Dose-dependent inhibition of MIP-1 β and TNF- α release from PBMCs when dendrimer glucosamine was added to human PBMCs for 1 h before LPS. The IC_{50} was $92 (6.8 \mu M) \pm 15 \mu g/ml$ ($n = 4$). Gray bars, PBMCs; black bars, PBMCs + LPS; white bars, PBMCs + dendrimer glucosamine + LPS. (**c,d**) Dendrimer glucosamine inhibited LPS-mediated release of MIP-1 β and TNF- α from DCs and MDMs. $n = 4$; *** $P < 0.001$. Gray bars, cells; black bars, cells + LPS; white bars, cells + dendrimer glucosamine + LPS. (**e,f**) The effect of dendrimer glucosamine on LPS-mediated release of MIP-1 β and TNF- α from MDMs was reversible. MDMs were cultured with dendrimer glucosamine for 1 h, washed with PBS and LPS was then added 1, 2, 4 or 8 h later for a period of 21 h. $n = 4$; ** $P < 0.01$; *** $P < 0.001$. Gray bars, cells; black bars, cells + LPS; white bars, cells + dendrimer glucosamine + PBS wash + LPS. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; DG, dendrimer glucosamine; DC, dendritic cells; MDM, monocyte-derived macrophages.

Table 1 Effect of dendrimer glucosamine on LPS-induced pro-inflammatory chemokine and cytokine mRNA synthesis in MDMs

Chemokines and cytokines	MDMs ^a	MDMs + LPS ^a	MDMs + DG + LPS ^a
MIP-1 α	14,438 \pm 1,438	178,375 \pm 6,125	21,925 \pm 1,450
MIP-1 β	13,938 \pm 1,563	328,750 \pm 11,750	30,125 \pm 625
IL-8	50,125 \pm 1,625	222,250 \pm 750	101,250 \pm 5,750
TNF- α	8,838 \pm 413	122,375 \pm 5,875	18,813 \pm 1,313
IL-1 β	1,163 \pm 213	183,125 \pm 33,875	10,588 \pm 263
IL-6	1,575 \pm 100	61,125 \pm 13,375	3,650 \pm 150

Results determined by quantitative, real-time Lightcycler PCR at 2 h.

^aCopies/ μ g RNA ($n = 4$). MDM, monocyte-derived macrophages; LPS, lipopolysaccharide; DG, dendrimer glucosamine.

dendrimer glucosamine did not interfere with LPS-induced maturation of DCs as determined by FACS analysis for CD25, CD80, CD83 and CD86 (Fig. 4a), or with β -interferon production (DCs + LPS 745 \pm 65 pg/ml versus DCs + dendrimer glucosamine + LPS 940 \pm 35 pg/ml; $n = 2$, $P = 0.16$; DCs alone 445 \pm 25 pg/ml). However, it did inhibit lymphocyte proliferation in the mixed leucocyte reaction (MLR) (Fig. 4b) and prevent γ -interferon production (206 \pm 24 versus 3 \pm 1 pg/ml; $n = 3$, $P < 0.001$). This effect was dose dependent with an IC₅₀ of 69 μ g/ml (5.1 μ M) \pm 11 μ g/ml (Fig. 4c).

The antiangiogenic activity of dendrimer glucosamine 6-sulfate

Dendrimer glucosamine 6-sulfate inhibited fibroblast growth factor-2 (FGF-2)-mediated proliferation of HUVECs by 50% \pm 3% at 50 μ g/ml (3.6 μ M) but had no effect on vascular endothelial growth factor (VEGF)-mediated proliferation of HUVECs (Fig. 5a). Its effect on new human blood vessel formation was also determined. In the Matrigel assay, dendrimer glucosamine 6-sulfate completely inhibited new microtubule formation by HUVECs at 100 μ g/ml (7.1 μ M) (Supplementary Table 1 online). In the human placental angiogenesis assay, it reduced new blood vessel formation by 50% at 50 μ g/ml (3.6 μ M) with the first significant effect seen at day 18. Maximum inhibition was seen at day 28 (Fig. 5b). Neither the dendrimer intermediate formed in the presence of the coupling agent only, nor glucosamine 6-sulfate nor the dendrimer had biological activity.

Therapeutic evaluation of dendrimer glucosamine conjugates

We used a validated and clinically relevant animal model of wound healing to establish whether an immuno-modulator (dendrimer glucosamine) and an antiangiogenic molecule (dendrimer glucosamine 6-sulfate) could act safely and synergistically to prevent scar tissue formation when used together (Fig. 5c). Subconjunctival scarring after glaucoma filtration surgery was chosen because (i) the surgical intervention is precisely defined (ii) it is one of the most aggressive models of excessive scar tissue formation available and (iii) surgical failure is due to excessive scar tissue formation. This animal model is better validated for scar formation in man than any other model of scarring^{17,18}; drug efficacy in this model has previously led to two new treatments that were effective in patients undergoing glaucoma surgery¹⁹, and to treatments for other scarring situations in man²⁰.

Glaucoma is the major cause of irreversible blindness worldwide. This surgical treatment is used in patients with glaucoma to create a new channel to drain aqueous humor from the eye. The subsequent reduction in intraocular pressure prevents the optic nerve damage that leads to blindness²¹. Post-surgical scarring is due to a persistent inflammatory and angiogenic response that promotes fibroblast

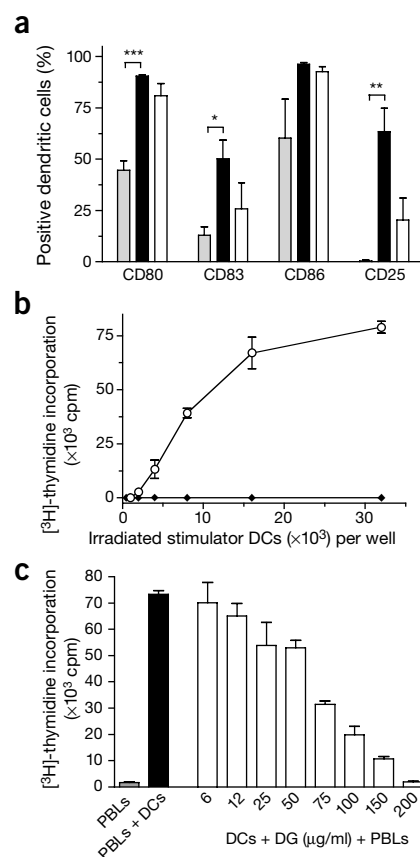


Figure 4 Effect of dendrimer glucosamine on dendritic cell-mediated functions. (a) LPS-induced expression of the cell surface-associated molecules CD80, CD83, CD86 & CD25 was not affected by dendrimer glucosamine. $n = 3$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Gray bars, DCs; black bars, DCs + LPS; white bars, DCs + dendrimer glucosamine + LPS. (b) The MLR was inhibited by dendrimer glucosamine. $n = 3$, $P < 0.001$. (○) DCs + PBLs; (◆) DCs + dendrimer glucosamine + PBLs. (c) The MLR dose-response curve for dendrimer glucosamine. The ratio of DCs/PBLs was 1:4. There was a reversible and dose-dependent effect of dendrimer glucosamine on the MLR with an IC₅₀ of 69 [5.1 μ M] \pm 11 μ g/ml. ($n = 4$). Gray bars, PBLs; black bars, PBLs + DCs; white bars, PBLs + dendrimer glucosamine + DCs. PBLs, peripheral blood responder lymphocytes; LPS, lipopolysaccharide; DG, dendrimer glucosamine; DC, dendritic cells; MLR, mixed leucocyte reaction.

proliferation²². This problem has led to the widespread use of the anticancer drugs 5-fluorouracil and mitomycin C to try and minimize scar tissue formation. However, their clinical use leads to sight-threatening complications that include thin, leaking tissues and severe infections²³.

We used the validated end point of bleb survival as our primary efficacy end point because this means that scar tissue formation has been significantly reduced and because this is indicative of the long-term patency of the new filtration pathway created (Supplementary Fig. 1 online). Combination treatment with dendrimer glucosamine and dendrimer glucosamine 6-sulfate increased the long-term success of the surgery from 30% to 80% ($P = 0.029$, Fig. 5d). The total dose of dendrimer glucosamine and dendrimer glucosamine 6-sulfate administered to each animal was 60.30 mg and 30.15 mg respectively. We saw no clinical, hematological or biochemical (including blood glucose) toxicity, and there were no local or systemic bacterial, viral or fungal infections in 30 rabbits treated over

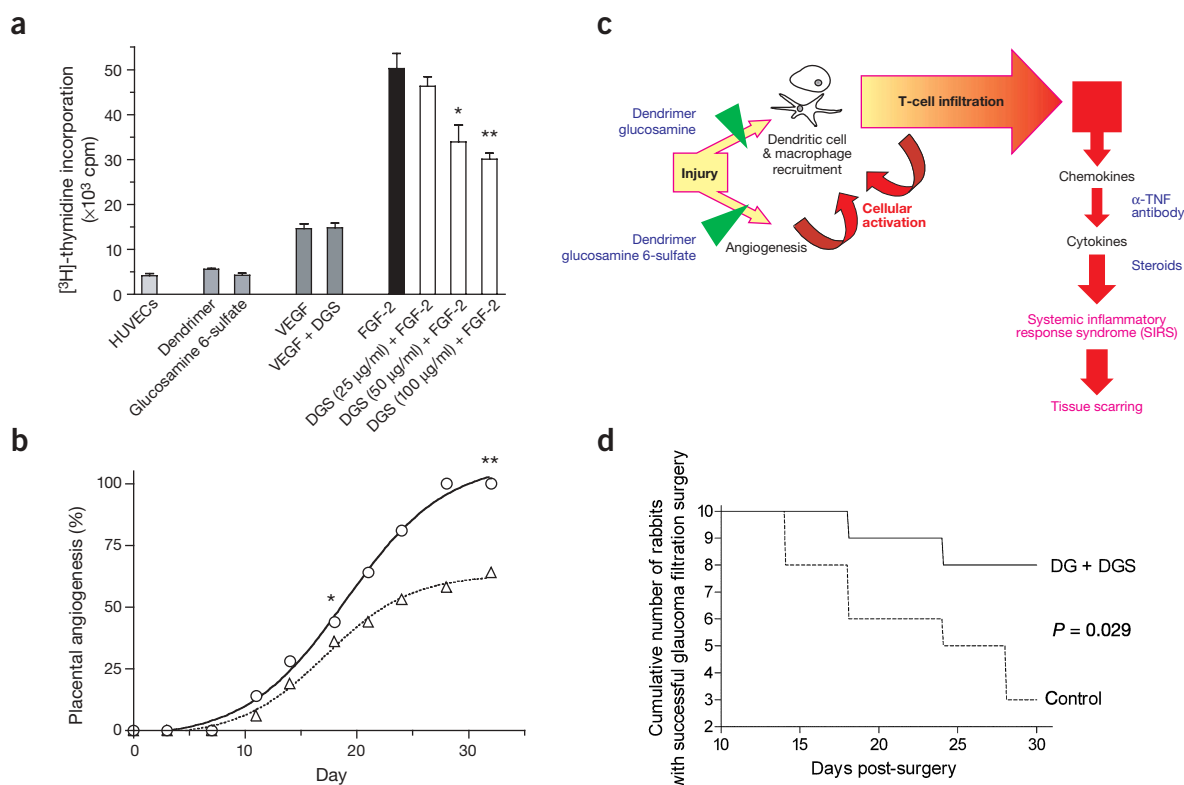


Figure 5 The anti-angiogenic activity of dendrimer glucosamine 6-sulfate. **(a)** Effect of dendrimer glucosamine 6-sulfate on VEGF and FGF-2-mediated proliferation of HUVECs. Dendrimer glucosamine 6-sulfate inhibited FGF-2-mediated proliferation of HUVECs by $50 \pm 3\%$ at $50 \mu\text{g/ml}$ [$3.6 \mu\text{M}$]. $n = 5$; $*P < 0.05$, $**P < 0.01$. **(b)** Dendrimer glucosamine 6-sulfate reduced the rate of new vessel formation in a human placental angiogenesis assay. Dendrimer glucosamine 6-sulfate (O). Media control (Δ). The results shown are for dendrimer glucosamine 6-sulfate at $50 \mu\text{g/ml}$ ($3.6 \mu\text{M}$). The graph is representative of two experiments done in quadruplicate. The degree of new vessel formation was quantified blind twice a week using a visual analog scale in which 0 = no growth, 1 = minimal new vessel formation, 2 = significant new vessel formation, and 3 = dense new vessel formation⁶. An angiogenesis score was derived from each count by dividing the total score (that is, the sum of all the wells) by the maximum possible score and then expressing the result as a percentage. A significant reduction in new vessel formation was first seen at day 18 ($*P < 0.05$). Maximum inhibition was seen at day 28 ($**P < 0.01$). **(c)** Illustration of the pathogenic mechanisms responsible for the systemic inflammatory response syndrome (SIRS) and scar tissue formation. The downstream sites of action of steroids and anti-TNF- α antibody are contrasted with the upstream sites of action of dendrimer glucosamine and dendrimer glucosamine 6-sulfate. **(d)** Cumulative graph of the number of rabbits with successful glaucoma filtration surgery. $P = 0.029$ (log rank test). Bleb survival at day 30 is indicative of the long-term patency of the surgical filtration pathway created⁵⁰. HUVECs, human umbilical vein endothelial cells; DG, dendrimer glucosamine; DGS, dendrimer glucosamine 6-sulfate; VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2.

30 d. Histopathological examination of the eyes from rabbits that were successfully treated with dendrimer glucosamine and dendrimer glucosamine 6-sulfate showed minimal scar tissue formation compared to placebo-treated animals (Fig. 6). In addition, there was no evidence of a persistent inflammatory or neoangiogenic response in successfully treated rabbits at day 30.

DISCUSSION

We have made and optimized two aminosaccharide dendrimer glycoconjugates and used them in combination to safely and synergistically prevent scar tissue formation after eye surgery. The dendrimer glucosamine conjugate acts by inhibiting LPS-TLR4 mediated pro-inflammatory mediator synthesis from immature human DCs and macrophages while allowing activation and maturation of DCs to occur. This is an important new property for a synthetic macromolecule because DC chemokine and cytokine secretion is central to the ability of these cells to orient and amplify specific components of the immune response. Other naturally occurring as well as synthetically derived macromolecules typically stimulate the release of pro-inflammatory cytokines through the TLR4 pathway and also induce

DC/macrophage maturation^{24–27}. The dendrimer glucosamine 6-sulfate conjugate inhibited FGF-2-mediated HUVEC proliferation and this translated into a significant antiangiogenic effect in two human model systems of new blood vessel formation. The combined use of dendrimer glucosamine and dendrimer glucosamine 6-sulfate increased the long-term success of glaucoma filtration surgery from 30% to 80% ($P = 0.029$). This study is the first demonstration that simultaneous targeting of pro-inflammatory mediators and neoangiogenesis with synthetic, water-soluble macromolecules with a narrow polydispersity that are neither toxic nor immunogenic, can safely and synergistically prevent scar tissue formation after surgery.

We used carboxylic acid-terminated PAMAM dendrimers because they are predominantly anionic at physiological pH. They are not toxic *in vitro* or *in vivo* compared to amine-terminated cationic PAMAM dendrimers. For example, cationic dendrimers cause substantial changes to red blood cell morphology at $10 \mu\text{g/ml}$ whereas anionic dendrimers have no such effect at $2,000 \mu\text{g/ml}$ ¹². Generation 3.5 anionic dendrimers were used because they are not toxic to mice at 95 mg/kg , and because they have a therapeutically useful pharmacokinetic profile¹². Glucosamine and glucosamine 6-sulfate were

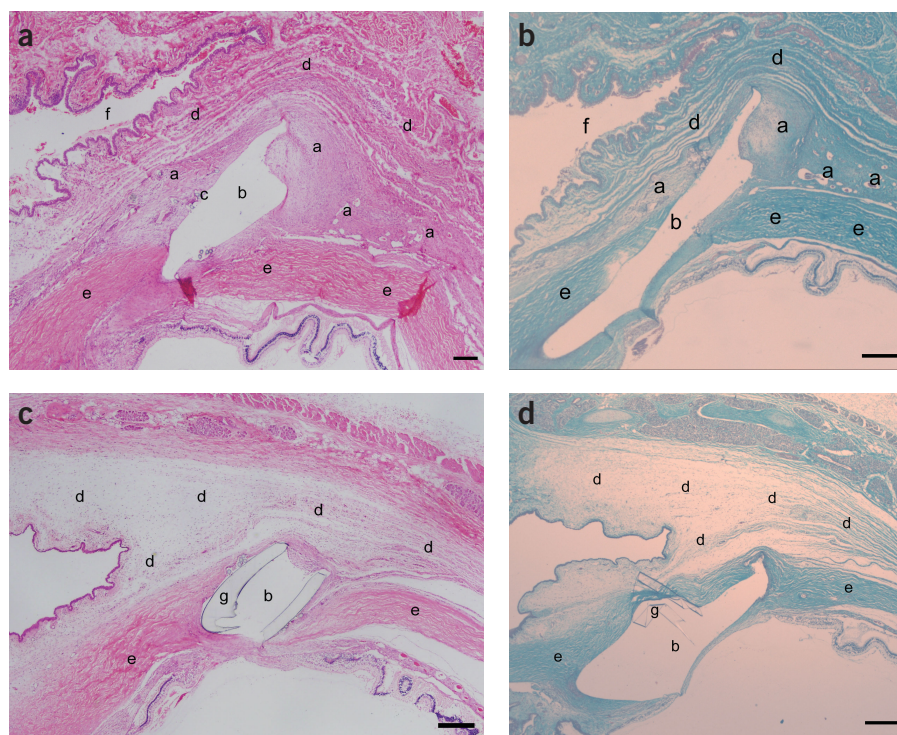


Figure 6 Histological cross-sections of rabbit eyes at day 30. (a,b) Histological cross-sections stained with haematoxylin & eosin and Masson's trichrome, respectively, from a control rabbit eye at day 30 after surgery. Masson's trichrome stains collagen green and muscle red. The sections show that the surgical drainage channel is surrounded by hypercellular scar tissue containing collagen. This extensive scar tissue formation prevents the drainage of aqueous humor across the conjunctiva and leads to the failure of the surgery. (c,d) Histological cross-sections stained with haematoxylin & eosin and Masson's trichrome, respectively, from a dendrimer glucosamine and dendrimer glucosamine 6-sulfate treated rabbit eye at day 30 after surgery. The sections show that the surgical drainage channel and cannula are surrounded by loose connective tissue. There is minimal scar tissue formation. This means that aqueous humor can drain across the conjunctiva and that the surgery has been successful. Normal collagen fibrils are seen in the cornea in all sections. a, scar tissue; b, channel; c, stitch material; d, conjunctiva; e, cornea; f, fornix; g, cannula. Scale bars, 1 mm.

used because several studies have suggested that they may improve wound healing^{28–30}. Furthermore, glucosamine is not toxic when administered intravenously and diffuses rapidly from the blood stream into tissues^{31–33}. It is also, by far, the most important precursor for all of the modified sugars found in the glycosaminoglycans that make up the backbone of connective tissue proteoglycans. This is also the first demonstration that anionic dendrimer conjugates of aminosaccharides can be made using water-based reactions rather than organic solvents. As such, it has important implications for their potential manufacture as pharmaceutical products.

The dendrimer glucosamine conjugate inhibited the synthesis of pro-inflammatory chemokines and cytokines from LPS treated DCs and MDMs (Fig. 3c,d and Table 1). These dose-dependent effects are consistent with dendrimer glucosamine having an inhibitory effect on the TLR4 that mediates LPS-induced stimulation of DCs and macrophages. This leads to an alteration of the immunological microenvironment that is sufficient to prevent leucocyte proliferation in a MLR (Fig. 4b) while allowing upregulation of costimulatory molecules (Fig. 4a). Our results suggest that costimulatory molecule upregulation can still occur because β -interferon production in response to LPS is not impaired by dendrimer glucosamine. This may reflect the distinct signal transduction pathways that regulate

chemokine/cytokine production (that is, TIRAP and MyD88) and β -interferon production (that is, TRAM and TRIF). Taken together, our results are consistent with a cell surface-mediated mechanism of action of dendrimer glucosamine that does not disrupt the cytoplasmic processing or trafficking of proteins to the cell surface^{34,35}.

Although pattern recognition receptors on DCs and macrophages have been primarily studied because of their role in the identification of exogenous molecules (e.g., LPS), it has recently become clear that the same innate immune mechanisms can also be triggered by surgically mediated tissue injury^{36,37}. In this context, our results suggest that inhibition of pro-inflammatory chemokine and cytokine release from DCs and macrophages by dendrimer glucosamine can be sufficient to interrupt the inflammatory, proliferative, maturation and remodeling phases of wound healing³⁸ that lead to excessive scar tissue formation (Fig. 5c). They also suggest that inhibiting pro-inflammatory chemokine release can abort the sequential release of pro-inflammatory cytokines (Fig. 2). The relevance of this observation is that MIP-1 α and MIP-1 β (i) are the first chemokines to be released by LPS^{15,16} (ii) their release is triggered via TLR4 following the deposition of fibrinogen in the earliest stages of an inflammatory response³⁹ and (iii) they are the most important mediators of the corneal inflammation and scarring that leads to blindness after herpes simplex type-1 infection⁴⁰.

The dendrimer glucosamine 6-sulfate conjugate has an inhibitory effect on the three most important hallmarks of angiogenesis in man; that is (i) FGF-2 receptor-mediated endothelial cell proliferation and migration (Fig. 5a)

(ii) capillary sprouting by normal endothelial cells (Supplementary Table 1 online) and (iii) new blood vessel formation in a model of cell migration and microvessel formation^{6,41–43} (Fig. 5b). Evaluation of antiangiogenic activity in adult animals is difficult because endothelial cells proliferate very slowly, if at all, under normal conditions. However, once a pathological stimulus intervenes, the pro-inflammatory and pro-angiogenic chemokine IL-8 activates endothelial cells, increases adhesion molecule expression, and promotes infiltration by inflammatory cells. These include antigen-activated CD4+ T lymphocytes which stimulate macrophages to produce TNF- α , IL-1 and IL-6 (ref. 44). This completes a positive feedback loop that leads to endothelial cell migration, division and reorganization^{45,46}. Our results show that the combination of dendrimer glucosamine and dendrimer glucosamine 6-sulfate acts synergistically to inhibit these very early and critical stages of the pro-inflammatory and pro-angiogenic responses (Fig. 5c). The preferential accumulation of dendrimer-based conjugates in tissues containing inflammatory cells has the added advantage of amplifying this pharmacological synergy³.

These synthetically engineered, narrow molecular weight aminosaccharide dendrimer conjugates are therefore an important

new class of therapeutically useful macromolecules for preventing scar tissue formation. They inhibit the polyvalent and cooperative cell surface receptor-ligand interactions that lead to TLR4 and fibroblast growth factor receptor-mediated signalling. As these two receptors play an important role in the pathogenesis of inflammation and angiogenesis respectively in a variety of diseases, dendrimer glucosamine and dendrimer glucosamine 6-sulfate could have clinically useful applications in other areas of medicine and surgery. For example, it is becoming clear that the molecular mechanism responsible for the LPS-TLR4 mediated syndrome of bacterial sepsis is also responsible for mediating the systemic inflammatory response syndrome (SIRS) in other shock-related diseases⁴⁷. Therefore, aminosaccharide dendrimer conjugates could be used to reduce the mortality associated with SIRS in patients after major surgical procedures, burns, acute pancreatitis and in bacterial sepsis. In the case of the last example given, we have unpublished data to show that the synthesis of pro-inflammatory chemokines and cytokines is significantly reduced by dendrimer glucosamine in human blood exposed to *Escherichia coli*. It will now require further detailed studies in appropriate and clinically validated models for each disease to determine the broader applicability of this therapeutic approach.

METHODS

Use of human blood cells was approved by the ethics committee of Hammersmith Hospitals Trust. Procedures involving animals were carried out in accordance with the UK Animals (Scientific Procedures) Act 1976.

Synthesis of glycoconjugate. In brief, Starburst PAMAM dendrimer generation 3.5 (304 mg, -ONa form, MW = 12,931, Dendritech) was allowed to react with D(+)-glucosamine hydrochloride (134 mg, Sigma-Aldrich), or D(+)-glucosamine 6-sulfate (161 mg, Dextra Laboratories) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI; 557 mg, Lancaster Synthesis) in water at pH 5. Dendrimer glucosamine and dendrimer glucosamine 6-sulfate were purified over Sephadex G-25 (Amersham Biosciences). Endotoxin was removed using Detoxi-Gel columns (AffinityPak Detoxi-Gel, Pierce) so that dendrimer glucosamine and dendrimer glucosamine 6-sulfate contained <0.06 endotoxin unit/ml (that is, European Community standard for water for injection) as determined using the *Limulus polyphemus* amebocyte lysate assay (Pyrotell). Full details are given in the Supplementary Notes.

Cellular toxicity. Dendrimer glucosamine and dendrimer glucosamine 6-sulfate were cultured with the human T-cell line, Sup-T1 and the human monocytic cell line, U937, for 67 h. PBMCs, MDMs, DCs and HUVECs were also cultured with dendrimer glucosamine and dendrimer glucosamine 6-sulfate for up to 5 d and their effect on cell growth and viability determined by cell count (Coulter ZM), trypan blue exclusion and the MTT assay⁴⁸.

Anticoagulant activity was determined by measuring the prothrombin time, which uses the extrinsic coagulation system, and the kaolin partial thromboplastin time, which uses the intrinsic coagulation system. Heparin-like activity was determined by measuring Factor Xa activity. Complement-activating activity was determined by measuring C3 and C4.

Lipopolysaccharide-induced chemokine and cytokine release. PBMCs, MDMs, immature DCs and CD3+ T lymphocytes were cultured in growth medium containing RPMI 1640, 330 µg/ml L-glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and 10% heat-inactivated human serum (Gibco). We cultured 8×10^5 cells/ml with dendrimer glucosamine (200 µg/ml) for 1 h at 37 °C and LPS (10 ng/ml; *Salmonella minnesota*, Sigma L9764) added. Supernatants were harvested 21 h later for MIP-1α, MIP-1β, IL-8, TNF-α, IL-1β, IL-6 and IFN-β (enzyme immunoassay (EIA), R & D Systems). mRNA was harvested at 2 h and measured by real time PCR using a Roche Lightcycler. Details of the PCR methods used are given in Supplementary Methods and Figure 2 online.

Dendrimer glucosamine did not bind to LPS or interfere with any of the enzyme immunoassays used. In other experiments, the cells were first cultured

with LPS and dendrimer glucosamine added 2 h or 4 h later. The reversibility of the immuno-modulatory activity of dendrimer glucosamine was determined by culturing 5-d-old MDMs with dendrimer glucosamine for 1 h, washing the cells with PBS, and adding LPS after 1, 2, 4 or 8 h. Culture supernatants were collected 21 h later.

DC assays. Immature DCs were prepared from human monocytes by culturing them for 5 d in growth medium containing IL-4 (1,000 U/ml) and granulocyte-macrophage colony stimulating factor (1,000 IU/ml). FACS analysis confirmed they were DCs (that is, CD3-, CD14-, CD16-, CD19-, HLA DR+) with an immature phenotype (that is, CD25-, CD80-, CD83-, CD86-). LPS lead to their maturation as defined by FACS analysis.

The effect of dendrimer glucosamine on LPS-induced maturation of DCs was determined by exposing cells (1×10^6) to dendrimer glucosamine (200 µg/ml) for 1 h followed by culture with LPS (10 ng/ml) for 21 h and FACS analysis. The effect of dendrimer glucosamine on the MLR was determined using [³H]-thymidine by culturing γ-irradiated stimulator DCs with dendrimer glucosamine (200 µg/ml) for 1 h and then adding allogeneic peripheral blood responder lymphocytes (100,000/well) for 5 d. γ-interferon and β-interferon were measured by EIA and mRNA PCR.

Neoangiogenesis assays. HUVECs were plated (passage 3; 2.5×10^4 cells/ml) on gelatin-coated plates in M199 medium (Invitrogen) supplemented with 20% human serum and heparin (50 µg/ml). Dendrimer glucosamine 6-sulfate (25–100 µg/ml) was added for 1 h followed by FGF-2 (10 ng/ml; Peprotech) or VEGF (20 ng/ml, R & D Systems) for 3 d. Cell proliferation was determined using [³H]-thymidine. A Matrigel assay was used to determine the effect of dendrimer glucosamine 6-sulfate on microtubule formation^{6,49}. HUVECs were plated (2.5×10^5 cells/ml) on tissue culture plates coated with Matrigel (Becton Dickinson) and dendrimer glucosamine 6-sulfate was added. The result was read at 18 h. For the placental angiogenesis assay, blood vessels (1–2 mm diameter) were excised from fresh human placentas, cut into fragments and cultured within a fibrin clot for 32 d. New vessel formation was quantified blind twice weekly by three observers⁶. The presence of endothelial cells in new vessels was confirmed using Factor VIII and CD31 antibodies.

Rabbit glaucoma filtration surgery model of excessive scar tissue formation.

Twenty female New Zealand white rabbits (2–2.4 kg, 12–14 weeks, Charles River) underwent experimental glaucoma filtration surgery¹⁷. A fornix-based conjunctival flap was raised behind the limbus and a scleral tunnel to the corneal stroma fashioned. A 22-G/25-mm venflon 2 cannula was passed through the tunnel until it was visible in the cornea. Entry into the anterior chamber was achieved by advancing the cannula to the mid-pupillary area where it was trimmed, beveled at its scleral end and fixed to the scleral surface with a nylon suture. The conjunctival incision was closed with two interrupted and one central mattress nylon suture. Dendrimer glucosamine (400 µg/ml) and dendrimer glucosamine 6-sulfate (200 µg/ml) were administered as a sub-conjunctival (100 µl) and intraperitoneal injection (10 ml) in a double-blind, placebo-controlled study on days –2, –1, 0, 1, 2, 3, 4, 7, 9, 11, 14, 17, 21, 24 and 28. The animals were evaluated twice weekly by masked observers. The primary efficacy end point was bleb survival because this is indicative of the long-term patency of the new filtration pathway created (Supplementary Fig. 1). Bleb failure was defined as the appearance of a vascularized, scarred bleb in association with a deep anterior chamber.

All results are shown as the mean ± s.e.m. *P* values were calculated using a Student's *t*-test.

ACKNOWLEDGMENTS

This study was supported by grants to S.S. from the National Institutes of Health (1-R21-A144694-01), The Wellcome Trust (068309) and The Wolfson Foundation (PR/013217). P.K. is supported, in part, by Moorfields Trustees, The Michael & Ilse Katz Foundation and an Alcon Research Institute Award.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

Received 31 March; accepted 7 May 2004

Published online at <http://www.nature.com/naturebiotechnology/>

- Tomalia, D.A., Naylor, A.M. & Goddard, W.A. III. Starburst dendrimers: molecular level control of size, shape, surface chemistry, topology and flexibility from atoms to macroscopic matter. *Angew. Chem. Int. Edit.* **29**, 138–175 (1990).
- Esfand, R. & Tomalia, D.A. Laboratory synthesis of poly(amidoamine) (PAMAM) dendrimers. in *Dendrimer and Other Dendritic Polymers* (eds. Frechet, J.M.J. & Tomalia, D.A.) 587–604 (Wiley, New York, 2001).
- Malik, N., Evagorou, E.G. & Duncan, R. Dendrimer-platinate: a novel approach to cancer chemotherapy. *Anticancer Drugs* **10**, 767–776 (1999).
- Zanini, D. & Roy, R. Practical synthesis of starburst PAMAM α -thiosialodendrimers for probing multivalent carbohydrate-lectin binding properties. *J. Org. Chem.* **63**, 3486–3491 (1998).
- Cloninger, M.J. Biological applications of dendrimers. *Curr. Opin. Chem. Biol.* **6**, 742–748 (2002).
- Thornton, M., Barkley, L. & Shaunak, S. The anti-Kaposi's sarcoma and anti-angiogenic activity of sulphated dextrans. *Antimicrob. Agents Chemotherapy* **43**, 2528–2533 (1999).
- Duncan, R. The dawning of polymer therapeutics. *Nat. Rev. Drug Discov.* **2**, 347–360 (2003).
- Roy, R. Recent developments in the rational design of multivalent glycoconjugates. *Top. Curr. Chem.* **187**, 24–274 (1997).
- Mammen, M., Choi, S.-K. & Whitesides, G.M. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Edit.* **37**, 2754–2794 (1998).
- Roberts, J.C., Bhalgat, M.K. & Zera, R.T. Preliminary biological evaluation of polyamidoamine (PAMAM) starburst dendrimers. *J. Biomed. Mater. Res.* **30**, 53–65 (1996).
- Jevprasesphant, R., Penny, J., Attwood, D., McKeown, N.B. & D'Emmanuele, A. Engineering of dendrimer surfaces to enhance transepithelial transport and reduce cytotoxicity. *Pharm. Res.* **20**, 1543–1550 (2003).
- Malik, N. *et al.* Dendrimers: relationship between structure and biocompatibility *in vitro*, and preliminary studies on the biodistribution of ^{125}I -labelled polyamidoamine dendrimers *in vivo*. *J. Controlled Release* **65**, 133–148 (2002).
- Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S. & Shimeno, H. Oversulfation of fucoidan enhances its anti-angiogenic and anti-tumor activities. *Biochem. Pharmacol.* **65**, 173–179 (2003).
- Brandtzaeg, P. *et al.* Net inflammatory capacity of human septic shock plasma evaluated by a monocyte based target cell assay: identification of interleukin-10 as a major functional deactivator of human monocytes. *J. Exp. Med.* **184**, 51–60 (1996).
- Wang, Z.M., Liu, C. & Dziarski, R. Chemokines are the main pro-inflammatory mediators in human monocytes activated by *Staphylococcus aureus*, peptidoglycan, and endotoxin. *J. Biol. Chem.* **275**, 20260–20267 (2000).
- Le Naour, F. *et al.* Profiling changes in gene expression during differentiation and maturation of monocyte derived dendritic cells using both oligonucleotide microarrays and proteomics. *J. Biol. Chem.* **276**, 17920–17931 (2001).
- Mead, A.L., Wong, T.T.L., Cordeiro, M.F., Anderson, I.K. & Khaw, P.T. Evaluation of anti-TGF- β_2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. *Invest. Ophthalmol. Vis. Sci.* **44**, 3394–3401 (2003).
- Khaw, P.T., Doyle, J.W., Sherwood, M.B., Smith, M.F. & McGorray, S. Effects of intraoperative 5-fluorouracil or mitomycin C on glaucoma filtration surgery in the rabbit. *Ophthalmology* **100**, 367–372 (1993).
- Siriwardena, D. *et al.* Human anti-TGF- β_2 monoclonal antibody—a new modulator of wound healing in trabeculectomy. *Ophthalmology* **109**, 427–431 (2002).
- Spinks, R.L., Baker, S.N., Jackson, A., Khaw, P.T. & Lemon, R.N. The problem of dural scarring: a solution using 5-fluorouracil. *J. Neurophysiol.* **90**, 1324–1332 (2003).
- The AGIS investigators. The advanced glaucoma intervention study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration. *Am. J. Ophthalmol.* **130**, 429–440 (2000).
- Chang, L., Crowston, J.G., Cordeiro, M.F., Akbar, A.N. & Khaw, P.T. The role of the immune system in conjunctival wound healing after glaucoma surgery. *Surv. Ophthalmol.* **45**, 49–68 (2000).
- Wells, A.P., Cordeiro, M.F., Bunce, C. & Khaw, P.T. Cystic bleb formation and related complications in limbus versus fornix based conjunctival flaps in paediatric and young adult trabeculectomy with mitomycin-C. *Ophthalmology* **110**, 2192–2197 (2003).
- Flo, F.H. *et al.* Involvement of TLR2 and TLR4 in cell activation by mannuronic acid polymers. *J. Biol. Chem.* **277**, 35489–35495 (2002).
- Garcia-Ramallo, E. *et al.* Resident cell chemokine expression serves as the major mechanism for leucocyte recruitment during local inflammation. *J. Immunol.* **169**, 6467–6473 (2002).
- Sweeney, E.A., Lortat-Jacob, H., Priestley, G.V., Nakamoto, B. & Papayannopoulou, T. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells. *Blood* **99**, 44–51 (2002).
- Termeer, C. *et al.* Oligosaccharides of hyaluronan activate dendritic cells via toll-like receptor 4. *J. Exp. Med.* **195**, 99–111 (2002).
- Lehto, M. & Jarvinen, M. Collagen and glycosaminoglycan synthesis of injured gastrocnemius muscle in rat. *Eur. Surg. Res.* **17**, 179–185 (1985).
- Fukazawa, M., Bryant, S.M., Orita, H., Campeau, J.D. & DiZerega, G.S. Modulation of proline and glucosamine incorporation into tissue repair cells by peritoneal macrophages. *J. Surg. Res.* **46**, 166–171 (1989).
- Schmidt, R.J., Spyrtou, O. & Turner, T.D. Biocompatibility of wound management products: the effect of various monosaccharides on L929 and 2002 fibroblast cells in culture. *J. Pharm. Pharmacol.* **41**, 781–784 (1989).
- Setnikar, I., Giachetti, C. & Zanol, G. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [^{14}C]glucosamine to the rat. *Pharmatherapeutica* **3**, 538–550 (1984).
- Aghazadeh-Habashi, A., Sattari, S., Pasutto, F.M. & Jamali, F. Single dose pharmacokinetics and bioavailability of glucosamine in the rat. *J. Pharm. Pharm. Sci.* **5**, 181–184 (2002).
- Setnikar, I. & Rovati, L.C. Absorption, distribution, metabolism and excretion of glucosamine sulfate. *Arzneimittelforschung* **51**, 699–725 (2001).
- Hoshino, K. *et al.* TLR4 deficient mice are hyporesponsive to LPS: evidence for TLR4 as the LPS gene product. *J. Immunol.* **162**, 3749–3752 (1999).
- Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443–451 (1999).
- Gordon, S. Pattern recognition receptors: doubling up for the innate immune response. *Cell* **111**, 927–930 (2002).
- Paterson, H.M. *et al.* Injury primes the innate immune system for enhanced toll-like receptor reactivity. *J. Immunol.* **171**, 1473–1483 (2003).
- Gillitzer, R. & Goebeler, M. Chemokines in cutaneous wound healing. *J. Leukocyte Biol.* **69**, 513–521 (2001).
- Smiley, S.T., King, J.A. & Hancock, W.W. Fibrinogen stimulates macrophage chemokine secretion through TLR4. *J. Immunol.* **167**, 2887–2894 (2001).
- Tumpey, T.M. *et al.* Absence of MIP-1 α prevents the development of blinding herpes stromal keratitis. *J. Virol.* **72**, 3705–3710 (1998).
- Pye, D.A., Vives, R.R., Turnbull, J.E., Hyde, P. & Gallagher, J.T. Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity. *J. Biol. Chem.* **273**, 22936–22942 (1998).
- Lundin, L. *et al.* Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis. *J. Biol. Chem.* **275**, 24653–24660 (2000).
- Schlessinger, J. *et al.* Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* **6**, 743–750 (2000).
- Li, A., Dubey, S., Varney, M.L., Dave, B.J. & Singh, R.K. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J. Immunol.* **170**, 3369–3376 (2003).
- Girard, J.P. & Springer, T.A. High endothelial venules: specialised endothelium for lymphocyte migration. *Immunol. Today* **16**, 449–457 (1995).
- Choy, E.H. & Panayi, G.S. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N. Engl. J. Med.* **344**, 907–916 (2001).
- Johnson, G.B., Brunn, G.J. & Platt, J.L. An endogenous pathway to systemic inflammatory response syndrome (SIRS)-like reactions through Toll-like receptor 4. *J. Immunol.* **172**, 20–24 (2004).
- Sgouras, D. & Duncan, R. Methods for the evaluation of biocompatibility of soluble synthetic polymers which have potential for biomedical use. 1. Use of tetrazolium-based colorimetric assay as a preliminary screen for evaluation of *in vitro* cytotoxicity. *J. Mater. Sci. Mater. Med.* **1**, 61–68 (1990).
- Kubota, Y., Kleinman, H.K., Martin, G.R. & Lawley, T.J. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* **107**, 1589–1598 (1988).
- Cordeiro, M.F., Gay, J.A., & Khaw, P.T. Human anti-TGF- β antibody: a new glaucoma anti-scarring agent. *Invest. Ophthalmol. Vis. Sci.* **40**, 2225–2234 (1999).