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Research Paper

Changes in Gene Expression Pattern of Human Primary Macrophages Induced by Carbosilane Dendrimer 2G-NN16

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Purpose. The use of dendrimers for biomedical applications has emerged with promising results. 2G-NN16 is a carbosilane dendrimer with sixteen positive charges per molecule tested to be capable to bind and release antisense oligonucleotides (ODNs) and small interference RNA (siRNA) *in vitro*. In spite of low cytotoxicity observed for these dendrimers, little is known about cellular changes they produce in cells in general and in immune cells in particular.
Materials and Methods. Genomic technologies allow us to identify global gene expression profile changes in macrophages exposed to a non-toxic concentration (5 μM) of 2G-NN16, alone or complexed with a random siRNA (dendriplex). Results were confirmed by quantitative real-time RT-PCR.
Results. Exposing macrophages to this dendrimer or dendriplex causes multiple gene expression changes, but no specific action of random siRNA was detected. Pathway analysis of differentially expressed genes shows the altered functions to be immune response, proliferation and transcription regulation. Interleukin 17F (IL17F) was the most regulated gene.
Conclusions. Global gene expression profiles are a highly sensitive method to measure the toxicity degree of a gene delivery vehicle. The strong repression of IL17F, IL23R and IL23A, all of which are involved in autoimmune disease, by this particular dendrimer suggests a potential pharmacological application.

KEY WORDS: carbosilane dendrimers; IL17F; macrophages; microarrays; siRNA.

INTRODUCTION

Nanotechnology, in general, is experiencing a rapid growth period with major advances arriving quickly. Accordingly, these advances are applied in the biomedical field in numerous and diverse ways (1). Dendrimers have been widely studied for biomedical applications. One of the most promising is the use of these compounds as potential vectors for targeted delivery of drugs, peptides, antisense oligonucleotides and siRNA (2,3). The principal advantage of their

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ABBREVIATIONS: 2G-NN16, $2G-\left[Si\left\{O(CH_2)_2N(Me)_2^+(CH_2)_2NMe_3^+(I^-)2\right\}\right]_8$; ALDOA, aldolase A, fructose-bisphosphate; AOC3, amine oxidase, copper containing 3; C18ORF10, chromosome 18 open reading frame 10; CCND3, cyclin D3; CCR1, chemokine (C–C motif) receptor 1; CCR2, chemokine (C–C motif) receptor 2; CD74, CD74 antigen; CR2, complement component (3d/Epstein Barr virus) receptor 2; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C–X–C motif) ligand 2; G-CSF, granulocyte colony-stimulating factor; H3F3B, H3 histone, family 3B; HIST1H4B, histone cluster 1, H4b; IL17F, Interleukin 17F; IL1B, Interleukin 1B; IL2, Interleukin 2; IL23A, Interleukin 23A; IL23R, Interleukin 23 Receptor; IL6, Interleukin 6; IL8, Interleukin 8; INF-γ, Interferon gamma; IPA, Ingenuity Pathways Analysis; LDH, Lactate dehydrogenase; MDK, midkine (neurite growth-promoting factor 2); MIP1-a, Macrophage inflammatory protein 1a; MIP1-b, Macrophage inflammatory protein 1b; MLLT6, myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 6; NFKB1A, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; ODNs, Oligonucleotide antisense; PAMAM, polyamidoamine dendrimers; PBMCs, peripheral blood mononuclear cells; PI, propide iodide; PRDX2, peroxiredoxin 2; PTMA, prothymosin, alpha; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, small interference RNA; SLC1A2, arrier family 1 (glial high affinity glutamate transporter), member 2; SYVN1, synovial apoptosis inhibitor 1, synoviolin; TIA1, TIA1 cytotoxic granule-associated RNA binding protein; TNF-α, tumor necrosis factor, alpha.

use is to improve the efficiency of the delivery by minimizing the amount of transported agent and the adverse reactions associated with the treatment. For example, introduction of long (>30 mer) double-strand RNA into mammalian cells leads to the initiation of the antiviral interferon response and global protein expression shutdown (4). Furthermore, the possibility to direct the drug to specific cell types by complexing the dendrimer with a specific antibody has been demonstrated (5). Another biomedical application is their use in imaging, for example (6) by monitoring the cardiovascular system, liver or kidney function and for imaging tumor vasculature (7).

The mononuclear phagocytic system, principally macrophages, plays a central role in the host defence system. In the same way as others foreign particles, dendrimers are first captured by cells of the mononuclear phagocytic system when circulating in blood (8). The interaction of macrophages with dendrimers is for this reason extremely important and justifies the investigation of the potential use of dendrimers as vehicles for drug delivery. Interactions between U-937 human macrophages and poly(propyleneimine) dendrimers have been recently described. In this work, these dendrimers at non-cytotoxic concentrations are shown to modify parameters such as intracellular responses-ROS content, mitochondria membrane potential, cell size and complexity, and cell cycle profiles (9).

Promising results have been obtained *in vitro* using 2G-NN16 carbosilane dendrimer as a vehicle for nucleic acids (6,10). In our laboratory, 2G-NN16 has been shown to be superior than commercial dendrimers, such as PAMAM (polyamidoamine) or superfect, when primary cell lines such as peripheral blood mononuclear cells (PBMCs) are transfected with siRNA. Toxicity assays (MTT, LDH, Trypan blue) in multiple primary and established cell lines have been conducted demonstrating a low effect on cellular viability at variable concentrations (6). However, toxicity assays studying how the delivery reagents affect gene expression are not routinely performed.

Two PAMAM dendrimers, differing only in their structural architecture, were shown to have opposite effects on endogenous epidermal growth factor receptor (EGFR) gene expression (11) in global gene expression profiling experiments. In the same work the authors also showed that PAMAM polyplexes with either DNA or siRNA produce different global changes in gene expression.

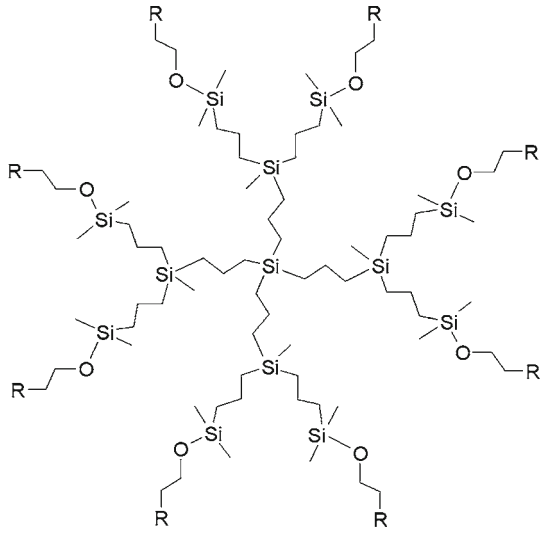
In the present work we analyze the changes in global gene expression profiles of primary cultures of human macrophages induced by exposure to 2G-NN16 carbosilane dendrimer. The effect of a dendriplex composed of 2G-NN16 and a random siRNA is also studied. Results obtained with microarrays for the gene IL17F and its regulator IL23R were confirmed and extended to IL23A using quantitative RT-PCR. Possible biomedical applications of direct exposure to 2G-NN16 dendrimer to primary human macrophages are discussed.

MATERIALS AND METHODS

Synthesis of $2G-[Si\{O(CH_2)_2N(Me)_2^+(CH_2)_2NME_3^+(I^-)_2\}]_8$ (2G-NN16) $2G-[Si\{O(CH_2)_2N(Me)_2^+(CH_2)_2NMe_3^+(I^-)_2\}]_8$, a carbosilane dendrimer, was used in the experiments. It is a second generation dendrimer that has a carbosilane skeleton and a cationic surface consisting of 16 quaternized amines distributed uniformly. It will be referred to as 2G-NN16 in this article for simplicity indicating that it is a second generation dendrimer with two peripheral nitrogen atoms per branch and sixteen positive charges. This dendrimer was synthesized as previously described and obtained as a white solid, soluble in water. 2G-NN16 contains eight Si-O bonds that gave it the ability to degrade along time in water (10). Full release of siRNA, previously complexed to the dendrimer, is observed at 24 h in a water solution. The hydrolysis of the carbosilane dendrimer used in this study is a slow process that takes place over a period of 12 to 24 h after being dissolved in water. Furthermore, the process of hydrolysis is retarded when the pH increases, so that in a physiological medium (pH about 7.4) the dendrimer remains mostly unaltered for at least 24 h. This is sufficient time to perform biomedical experiments such as toxicity profiles or transfection assays. Given that the time used in our experiments was 5 h, we can be certain that dendrimer and the dendriplex, were not degradated. The structure of this dendrimer is shown in Fig. 1.

Macrophages Isolation and Culture

PBMCs obtained from healthy individuals, were processed by Ficoll density gradient centrifugation and activated with 60 U/mL of IL2 and 2 μg/mL of phytohemaglutinine for 72 h in RPMI medium enriched with 10% of fetal bovine serum and 1% of ampicillin, 1% of cloxacillin, 0.32% of gentamicin and 2 nM glutamine at 37°C in a 5% CO_2 atmosphere. Macrophages were isolated from these cells mechanically, and were exposed to either 2G-NN16 dendrimer or 2G-NN16 complexed with a random siRNA (dendriplex), for 5 h in RPMI medium with the same



NN16: $R = N^+(CH_3)_2CH_2CH_2N^+(CH_3)_3$ **NN16:** R =

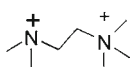


Fig. 1. Complete chemical structure of 2G-NN16 dendrimer. A total of 16 positive charges are present in the periphery of dendrimer resulting in the capacity to bind the negatively charged siRNA.

enrichment. For dendriplex formation, a ratio of charges 20:1 (NN16/siRNA) was used. The siRNA used was a negative control tested to have no effect on human samples (D-001910-02, Accell non-targeting siRNA, Thermo Fisher Scientific, Chicago).

Cytotoxicity Assays

Toxicity assays were performed from two different donors by measuring lactate dehydrogenase (LDH) and propide iodide (PI) (Fig. 2). For the LDH assay (2B), macrophages were grown at a concentration of 0.5×10^6 cells/well (approximately) where they were kept overnight before the treatment with the dendrimer or dendriplex. Cells were exposed to 1, 2.5 and 5 μM of 2G-NN16 dendrimer or dendriplex for 5 h. A parallel aliquot of cells was cultured in absence of dendrimer for 5 h as a control. The cells were detached mechanically, rinsed in PBS, and centrifuged at 2,500 rpm for 10 min. Supernatant was used to measure toxicity by LDH cytotoxicity, following the instructions of CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Absorbance was read using Anthos 2001 Microplate Reader (Inmunogenetics Diagnostica y terapeutica S.A.).

For the IP assays (2A), macrophages were grown in conditions similar to those used for the LDH assays. After addition of RPMI media and exposure to either dendrimer or dendriplex, macrophages were washed twice in cold 1× PBS, resuspended in 100 μl of 1× PBS and incubated in dark with 5 μl of α-CD5-PC5 and α-CD14-FITC (both from Beckman Coulter) for 30 min at room temperature. Cells were washed again in 1× PBS and incubated in dark with 5 μl of IP (1 mg/ml) solution for 30 min at room temperature. Finally, macrophages were analyzed in a FC500 flow cytometer (Beckman Coulter), determining the number of dead macrophages by IP staining.

RNA Extraction

Macrophages were purified and exposed to RPMI, 5 μM dendrimer or 5 μM dendriplex as described above. RNA from purified macrophages (approximately 4×10^6 cells for each condition) from three donors was extracted using RNeasy Mini Kit (Qiagen) following manufacture's instructions. RNA concentrations were measured using the Nanodrop Spectrophotometer ND-100 UV/Vis (Nanodrop Technologies). When necessary, RNA was concentrated using a Concentrator 5301 (Eppendorf). Before carrying out the microarray chip assays, RNA integrity was verified using Agilent RNA 6000 Nano Kit in a 2100 Bioanalyzer (Agilent Technologies).

Microarrays

Microarrays experiments were performed following the MIAME (Minimal information About a Microarry Experiment) criteria. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (12) and are accessible through GEO Series accession number GSE12405 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12405). For each sample, 1 μg of total RNA was amplified and labelled using SuperScript Indirect RNA Amplification Kit (Invitrogen) following manufacture's instructions. Briefly, RNA was denatured and reverse transcribed to cDNA. This cDNA was purified and *in vitro* transcribed to RNA using T7 RNA polymerase. Amplified RNA was purified and quantified using a Nanodrop Spectrophotometer ND-100 UV/Vis (Nanodrop Technologies). Control and dendrimer probes were labelled, respectively, with Alexa Fluor 647 reactive dye decapack and Alexa Fluor 555 reactive dye decapack. Labeled RNA was again purified and quantified using a Nanodrop Spectrophotometer ND-100 UV/Vis (Nanodrop Technologies) and RNA quality checked using the Agilent 2100 Bioanalyzer (Agilent Technologies).

Before the hybridization, RNA (1.65 μg) was fragmented using the Gene Expression Hybridization Kit (Agilent Technologies) by incubation with 25× Fragmentation Buffer and 10× Blocking Agent for 30 min at 60°C. Fragmented amplified RNA was hybridized to Agilent 4×44 K whole genome human chip (Agilent Technologies). This multi-pack (4×44 K) formatted microarray represents a compiled view of the human genome as it is understood today. The sequence information used to design this microarray was derived from a broad survey of well-known sources such as RefSeq, Goldenpath, Ensembl, Unigene and others. The resulting view of the human genome includes probes for 41,000 unique genes and transcripts that have been verified and optimized by alignment to the human genome assembly and by Agilent's Empirical Validation process. Targets were hybridized in Hybridization Buffer of Gene Expression Hybridization kit using gasket slides (Hybridization Gasket Slide Kit—four microarrays per slide format, Agilent Technologies, CA), Agilent microarray hybridization chambers (G2534A) (Agilent Technologies) and a

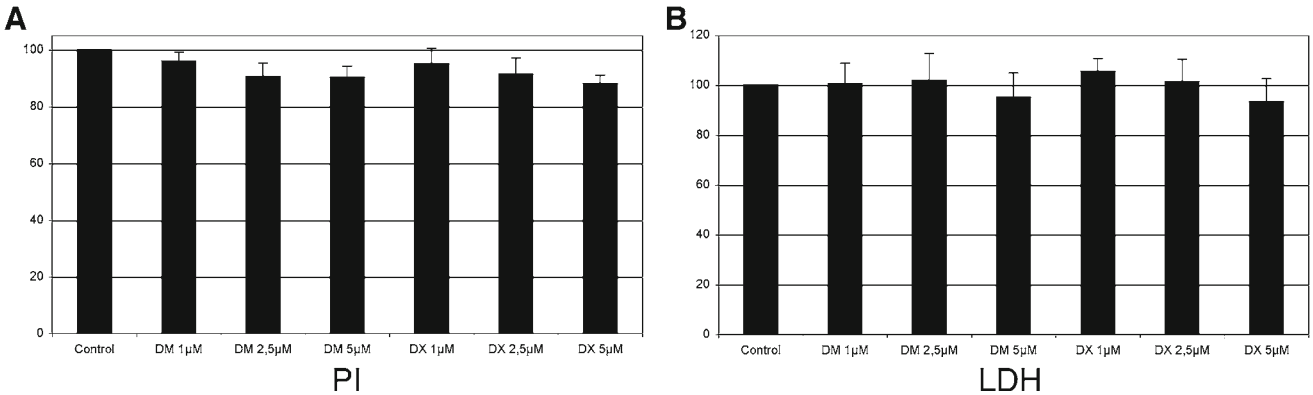


Fig. 2. Toxicity assays on macrophages by exposure at 1, 2.5 and 5 μM performed using PI in a flow cytometer (**A**) and LDH (**B**). Data were collected in duplicate.

DNA Microarray Hybridization Oven (Agilent Technologies) for 17 h at 65°C and 10 rpm. After this time, two washes of 1 min each, the first with Wash Solution 1 and the second with Wash Solution 2 (Agilent Technologies) were performed. The slides were centrifugated at 1,000 rpm for 2 min, before the scanning process. A total of six microarrays were used, three were hybridized for the Control *vs* Dendrimers comparison and three for the Control *vs* Dendriplex comparison, each corresponding to a different donor.

Slides were scanned at 5 μm resolution on a GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA) with independent excitation of the fluorophores Cy3 and Cy5. The resulting TIFF images were analyzed using GenePix Pro 4.0 software (Axon Instruments, Inc., Foster City, CA). Spots or areas of the array with obvious defects were manually flagged. The signal and background fluorescence intensities were calculated for each spot, and a GPR file was obtained for each hybridization. The files from the different experiments (four points/slide) were loaded into the MIAME compliant Almazen database software (http://almazen.bioalma.com). Background was subtracted and each experiment normalized by total intensity and sub-grid lowess. A gene was considered to be induced or repressed when the fold change was > 2 or <−2 and with a *p*-value of <0.05. As an additional filter, only genes with *z* scores of >1.8 or <−1.8, and average signal of >32 (to discard spots close to background) were selected for further statistical analyses.

Data Analysis

Potential signalling pathways were analyzed by using Ingenuity Pathways Analysis (IPA) (http://www.Ingenuity.com). This web-delivered application reveals relevant networks by comparing gene expression data with known signalling pathways. The filtered gene expression data set of cells treated with dendrimer or cells treated with dendriplex were uploaded as tab-delimited text files into the IPA for generating biological networks. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. This software then assigned a score for all networks that were ranked on the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone (a score of 2 represents a 99% confidence level, and 3 a 99.9%). Biological functions are then calculated and assigned to each network.

Quantitative Real-Time RT-PCR

cDNA was generated from 200 ng of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems) in a 10 μL final reaction volume following manufacture's instructions. Real-time PCR was performed using 3 μL/well of two serial dilutions performed in triplicate for 1/50 dilution and duplicate for 1/5,000 dilution of each cDNA, 0.03 μM 18S, IL17F, IL23R or IL23A (18S forward, 5′-GCA ATT ATT CCC CAT GAA CG-3′; 18S reverse, 5′-GGG ACT TAA TCA ACG CAA GC-3′; IL17F forward, 5′-GGC ATC ATC AAT GAA AAC CA-3′; IL17F reverse, 5′-TGG GGT CCC AAG TGA CAG-3′; IL23R, forward, 5′-CCA TCT CTA CAG GGC ACC TTA C-3′; IL23R reverse, 5′-CGA TCA TTC CCA ATA AAA GTC C-3′; IL23A forward, 5′-TGT TCC CCA TAT CCA GTG-3′; IL23A reverse 5′-TCC TTT GCA AGC AGA ACT GA- 3′), 1× SYBR Green PCR Master Mix in 8 μL in MicroAmpTM Optical 384-well reaction plate (Applied Biosystems, Foster City). PCR reactions were run on an Applied Biosystems ABI PRISM 7900 HT and SDS v2.2 software was used to analyze the results with the Comparative Ct Method (ΔΔCt).

RESULTS

PI and LDH Toxicity

Toxicity measurements by PI (Fig. 2A) and LDH (Fig. 1B) assays were performed with purified macrophages exposed to 1, 2.5 and 5 μM concentration of either 2G-NN16 dendrimer or 2G-NN16 complexed with a random siRNA (20:1 molecules ratio). Both assays identified a slight, but dose-dependent, toxicity. The dendriplex at 5 μM produced cell mortality of 12%, as determined by the PI assay. Analysis of these same conditions using the LDH assay indicated cell mortality of less than 10%, which is limit to be considered toxic. These results confirm our experiments using other cell types (data not presented) showing that 5 μM is the concentration limit above which significant mortality is observed.

Dendrímer *vs* Dendriplex Microarrays

Microarray analysis of gene expression profiles in the human transcriptome reveals that 4,785 genes were over-expressed or under-expressed (more than two times) in macrophages exposed to 2G-NN16 alone at 5 μM. When we analyze the number of genes 2-fold under- or over-expressed in macrophages exposed to the same concentration of 2G-NN16 dendrimer complexed with a random siRNA (dendriplex), we can see that 11,138 genes are affected. However, the number of replicates performed for each condition, three from different donors, let us filter these genes using criteria standard for microarray data: *p* value (<0.05), average signal (>32) and *Z*-score (>1.8) (13). After applying this filter, 65 genes have been selected as being differentially expressed in macrophages exposed to the dendrimer alone, against 271 genes when exposed to the dendriplex. In order to determine if these differences are specific to the dendrimer exposure or to the dendriplex exposure, scatter plots were generated comparing the LogRatio values in both conditions for the selected genes (Fig. 3). A logistic regression (R^2) for each list of genes was performed showing a strong correlation of data in dendrimer's selected genes (R^2=0.975) and in dendriplex (R^2=0.964). This concordance is even higher than that when all genes (R^2=0.86) are used. The tendency of regulation is similar for all genes selected, except for HIST1H4B, which was 4.4-fold over-expressed in dendriplex-exposed macrophages, but was not affected by dendrimer exposure. This indicates that genes affected by dendriplex exposure are also affected by dendrimer, but many of them have not been selected because one or more of the filtering criteria were not passed. A similar result is obtained when we analyze fold change values of genes selected by dendrimer exposure with the corresponding fold change values by dendriplex exposure. It is likely that a larger number of replicates would minimize

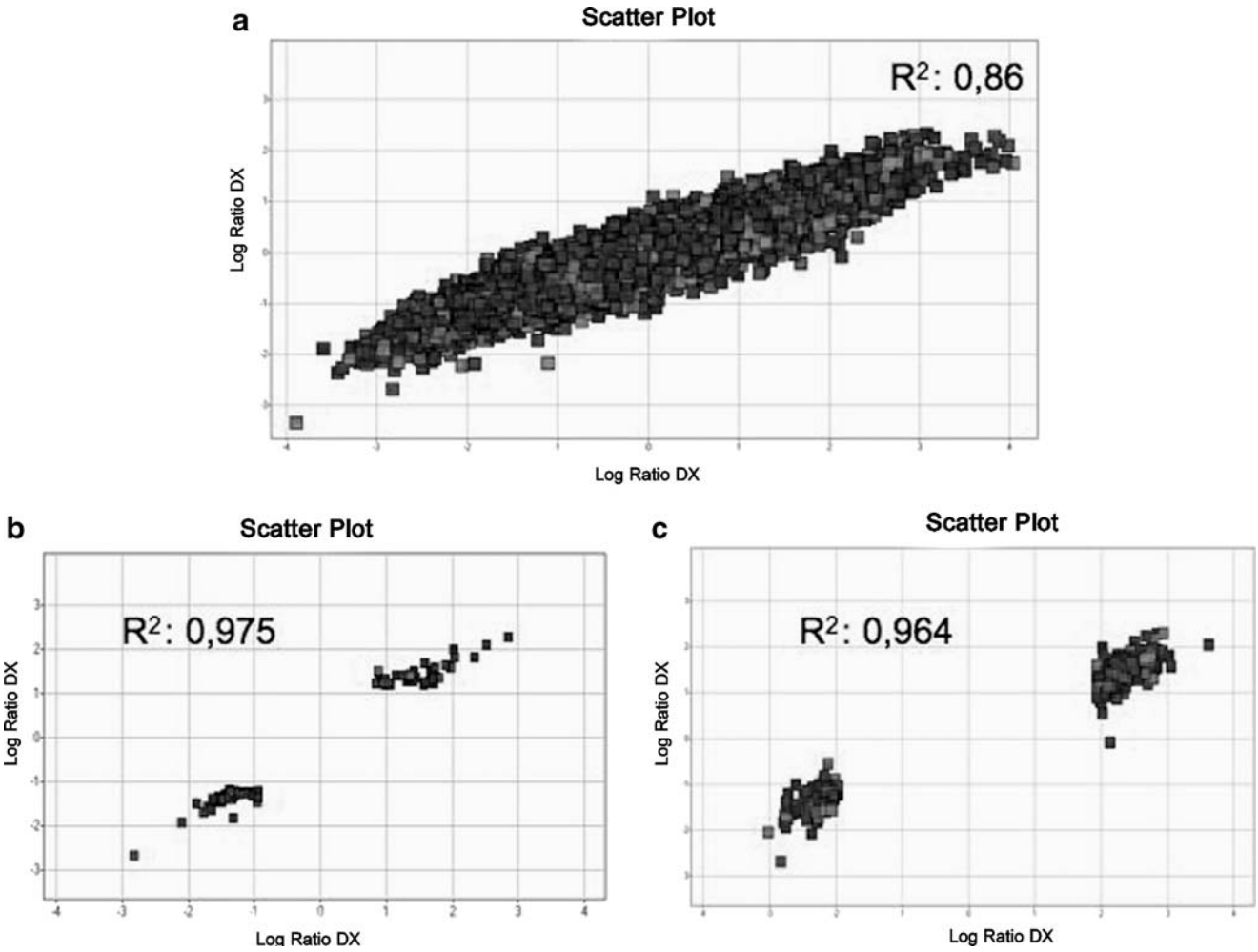


Fig. 3. **a** LogRatio Scatter Plot of macrophages exposed to 5 μM 2G-NN16 *versus* Dendriplex. Spots were filtered by Average intensity higher than 32 units in both conditions. Correlation factor (R^2) was 0,86, indicating a high level of correlation. **b** Scatter plot (dendrimer *vs* dendriplex) with significant selected genes differentially expressed in response to dendrimer exposure. **c** Scatter plot (dendrimer *vs* dendriplex) of significant genes differentially expressed in response to dendriplex exposure. In **b** and **c** a very high correlation is observed.

this variation. Analyzing these results, we obtain a unique list with the most significant differentially expressed genes in dendrimer and/or dendriplex exposed macrophages, which includes 331 different genes (Supplemental data; for a selection of the 50 most relevant regulated genes see Table I). Among these differentially expressed transcripts, we find genes involved in immune response, cancer, transcription regulation and viral function. The most statistically significant gene was Interleukin 17F (IL17F) (6.4-fold repressed and 7.1-fold repressed by dendrimer and dendriplex exposure, respectively, with a p value of 0.008 in both cases).

The list of genes was analyzed for most likely altered functions, interactions and pathways using IPA. This software identifies relationships between genes in the list by establishing networks based on interactions described in the literature. Functional annotations are provided for each gene in the list, as well as putative functions significantly altered by the treatment, based on the identity of the networks identified. Results obtained with respect to the functions with the highest probability to be represented in the gene list are summarized in Table II. The principal functions were cellular movement of blood cells and haematological system development and function (AOC3, CCR1, CCR2, CXCL1, IL1B) (p-Value from 9.05×10^{-6} to 6.98×10^{-6}), immune response, cell-to-cell signalling and interaction in recruitment of phagocytes (CCR1, CCR2, CR2, CXCL1, CXCL2, IL8, IL1B, MDK) (p value from 1.37×10^{-5} to 1.1×10^{-5}), connective tissue disorders and inflammatory diseases (arthritis) (ALDOA, C18ORF10, CCND3, CCR1, CCR2, CD74, CR2, CXCL1, CXCL2, H3F3B, IL8, IL1B, MDK, MLLT6, NFKBIA, PRDX2, PTMA, SLC1A2, SYVN1, TIA1) (p value 8.76×10^{-5}).

A graphic of the most significant network identified is shown (Fig. 4). Principal functions over-represented in this network are cancer, immune response and transcription regulation.

Quantitative Real Time RT-PCR

In order to validate the microarray results, quantitative real-time RT-PCR (qRT-PCR) was performed in quintupli-

Table I. List of the 50 Most Regulated Genes in Macrophages by 2G-NN16 and Dendriplex Exposition

List of genes selected by differential expression in macrophages by NN16 or dendriplex effect				
Symbol	Fold Change DM	*p* value DM	Fold Change DX	*p* value DX
IL17F	−6.45	0.0090	−7.11	0.0080
A_24_P247493	−4.23	0.1143	−5.14	0.0448
GALM	−4.14	0.1532	−8.12	0.0354
FADD	−3.84	0.1328	−6.75	0.0362
COPA	−3.80	0.0385	−4.28	0.0906
A_24_P118721	−3.55	0.0042	−2.50	0.1237
A_24_P75408	−3.55	0.1358	−6.91	0.0331
CCR1	−3.54	0.2851	−4.85	0.0475
ZMYM6	−3.42	0.1007	−5.42	0.0470
LOC642443	−3.37	0.1775	−6.55	0.0472
LOC732268	−3.33	0.0777	−4.79	0.0402
ENSA	−3.32	0.1381	−6.74	0.0189
X58329	−3.28	0.0137	−3.42	0.0144
A_24_P203984	−3.15	0.2048	−6.82	0.0485
A_24_P600622	−3.12	0.0344	−3.14	0.0558
LOC648992	−3.05	0.1656	−5.96	0.0352
ADH5	−3.04	0.2215	−6.56	0.0497
PRDX2	−3.03	0.1272	−4.97	0.0373
GRAP2	−3.01	0.0809	−4.46	0.0277
GSTK1	−2.98	0.1081	−4.64	0.0452
INTS4	−2.97	0.0339	−3.26	0.0546
XRCC4	−2.95	0.0908	−4.19	0.0459
CIP29	−2.95	0.0871	−4.14	0.0461
S80931	−2.94	0.1354	−4.68	0.0492
A_24_P118422	−2.93	0.0945	−4.24	0.0317
DUB3	3.53	0.1895	8.12	0.0309
BC041913	3.54	0.0747	4.74	0.0170
AK000420	3.54	0.0392	5.04	0.0053
PIM1	3.54	0.0296	4.09	0.0093
AK057167	3.57	0.0881	7.24	0.0017
PTMA	3.57	0.1496	5.66	0.0359
DOCK4	3.58	0.1241	6.85	0.0119
A_24_P551530	3.61	0.0760	5.74	0.0261
ANKHD1	3.69	0.1424	6.54	0.0405
ADCY7	3.72	0.1251	6.06	0.0445
AMPD3	3.73	0.1962	7.56	0.0315
ARRDC3	3.74	0.1651	5.43	0.0284
A_23_P136013	3.77	0.0636	5.40	0.0109
AK025704	3.78	0.1574	6.51	0.0316
A_32_P221641	3.82	0.0740	5.88	0.0248
ATP2A2	3.87	0.1676	6.87	0.0408
THC2476126	3.98	0.0360	4.06	0.0230
FAM40B	4.12	0.2207	12.30	0.0031
THC2545558	4.31	0.0268	5.68	0.0089
IL8	4.47	0.1281	6.80	0.0177
ARID4B	4.49	0.0938	6.91	0.0376
USP54	4.69	0.0837	6.39	0.0252
PCGF3	4.86	0.0476	7.18	0.0149
PCGF3	4.86	0.0476	7.18	0.0149
RSNL2	4.91	0.1047	7.69	0.0369

A full list can be viewed as supplemental table.

cate on a new sample for IL17F, the most significant regulated gene, using ribosomal 18s as a control for normalization. IL17F mRNA was clearly down-regulated in macrophages exposed to 2G-NN16 dendrimer (6.25-fold) as well as in macrophages exposed to dendriplex (23.25-fold) (Fig. 5). The degree of IL17F repression observed by qRT-PCR was greater than that measured using microarrays. This is often seen as qRT-PCR is a much more sensitive technique (14). Given this strong repression, we investigated the expression behaviour of IL23R and its ligand IL23, which are inducers of IL17F expression. IL23R was 3.65-fold repressed in macrophages exposed to 2G-NN16 and 2.19-fold repressed in macrophages exposed to dendriplex in microarray experiments. However, this gene did not pass the selection filter because, in the first case, the signal intensity was very low and, in the second, the p-value was greater

Table II. Results of Analysis using the Ingenuity Pathway Analysis Software Showing the Functions with the Highest Probability to be Affected in Macrophages by Dendrimer/Dendriplex Exposure

Category	Process annotation	Significance	Molecules
Cellular movement	Extravasation of blood cells	9.05E−06	AOC3, CCR1, CCR2, CXCL1, IL1B
Hematological system development and function	Extravasation of leukocytes	6.98E−06	AOC3, CCR1, CCR2, CXCL1, IL1B
Cell-to-cell signaling and interaction	Recruitment of phagocytes	1.37E−05	CCR1, CCR2, CR2, CXCL1, CXCL2, IL8, IL1B, MDK
Immune response	Recruitment of granulocytes	1.13E−05	CCR1, CCR2, CR2, CXCL1, CXCL2, IL8, IL1B, MDK
Inflammatory disease	Arthritis	8.76E−05	ALDOA, C18ORF10, CCND3, CCR1, CCR2, CD74, CR2, CXCL1, CXCL2, H3F3B, IL8, IL1B, MDK, MLLT6, NFKBIA, PRDX2, PTMA, SLC1A2, SYVN1, TIA1
Connective tissue disorders	Arthritis	8.76E−05	ALDOA, C18ORF10, CCND3, CCR1, CCR2, CD74, CR2, CXCL1, CXCL2, H3F3B, IL8, IL1B, MDK, MLLT6, NFKBIA, PRDX2, PTMA, SLC1A2, SYVN1, TIA1

Selected genes were included in Ingenuity to have this result. In the table, parameters such as Category, Process Annotation, Significance and Molecules are presented.

than 0.05. Given this result, we analyzed IL23R expression macrophages exposed to 2G-NN16 or dendriplex by qRT-PCR. IL23R, like IL17F, was down-regulated in both conditions (2.2-fold by 2G-NN16 exposure and 5.5-fold by dendriplex exposure (Fig. 5). In addition, we studied the expression of the p19 subunit of IL23, a protein subunit which is specific to the IL23 molecule. IL23 is a heterodimeric cytokine composed of the unique p19 subunit and a common p40 subunit which is also part of the Interleukin-12 molecule (15). Results confirmed a repression of 1.47-fold for dendrimer and 3.48-fold for dendriplex exposure.

DISCUSSION

The use of dendrimers in biomedicine as vehicles for drug delivery is rapidly increasing in the last years with much investigation currently in progress (16–18). Our group is working with carbosilane dendrimers that are truly spherical macromolecules constituted by an inert and lipophilic skeleton. They have several properties that make them attractive as drug target delivery systems. For example, the highly lipophilic structure may help to increase the biopermeability of these carbosilane systems compared to other dendrimers with different structures. One of these dendrimers in which we are interested—2G-NN16—is a molecule capable of binding to antisense oligonucleotides (ODNs), siRNAs, plasmids and peptides by a union that is degraded over a period of 24 h, resulting in a gradual release of the transported molecule. Moreover, the nucleic acid, when bound to the dendrimer, is protected from BSA binding, which allows higher effective concentrations of siRNA or ODNs to be maintained in the blood-stream (6).

Toxicological studies performed usually measure the percentage of death and little is known about other effects of dendrimers. Administration of *N*-acetyl-glucosamine-coated (GlcNAc8) PAMAM generation 1 glycodendrimers to mice bearing subcutaneous B16F10-induced melanoma decreased tumor growth and increased survival (19). Also, cytokines, including IL-1ß, INF-γ, TNF-α and IL2, showed increased production levels. The PAMAM generation 3.5-glucosamine dendrimers also showed immunomodulatory properties in human dendritic cells and macrophages, inducing synthesis of the pro-inflammatory cytokines MIP1-a, MIP-1ß, and IL-8, as well as the cytokines TNF-α, IL-1ß and IL-6 (20). Recently, it has been demonstrated by microarray analysis that treatment of the U87 established cell line with the 2G-NN16 dendrimer has no effect on gene expression (manuscript in preparation). As macrophage is one the first barriers of the immune system, we wanted to evaluate the effects of 2G-NN16 on primary cultures of human macrophages. No significant cell death was observed by either PI or LDH cytotoxicity assays in the presence of 5 μM of 2G-NN16 compared to the control. However, given that changes in gene expression profiles have been described for others dendrimers (21), microarray analyses were performed. The number of genes over-expressed or under-expressed 2-fold or greater was similar to the results obtained by Hollins *et al.* (11), given that we screened 44,000 instead of

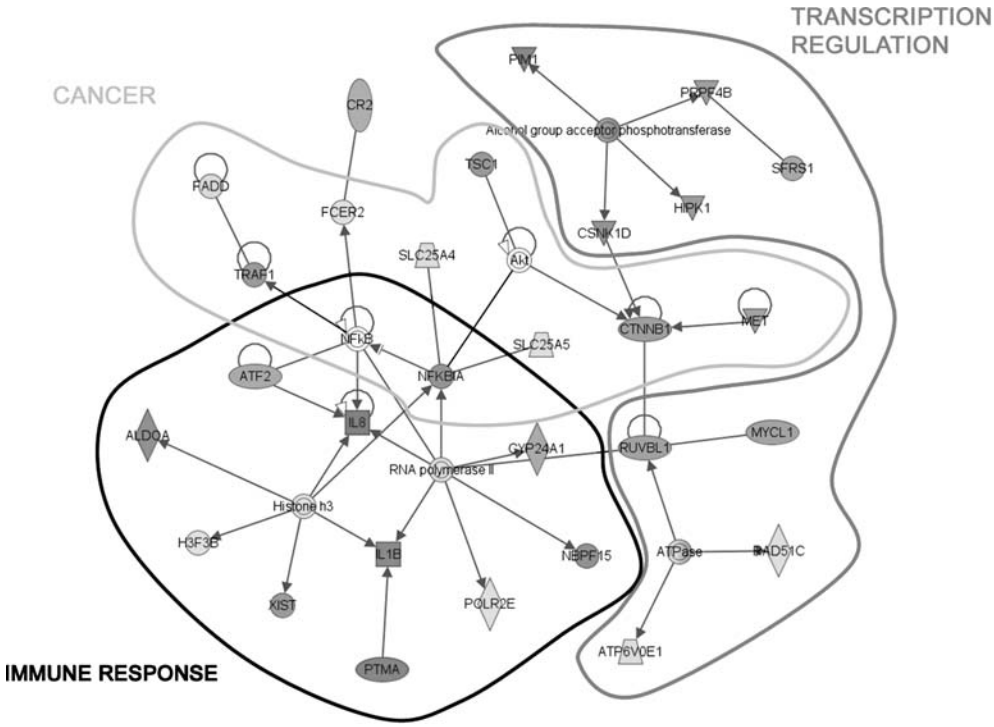


Fig. 4. Graphical representation of Network 1 introducing the list of selected genes in Ingenuity Pathways Analysis. Three principal functions are observed: immune system, cancer and transcriptional regulation.

22,000 transcripts. However, the application of more restrictive statistical criteria allows us to focus on 330 genes. Analysis of pathways in which these differentially expressed genes are know to function showed that immune response, cancer, transcription regulation and viral function are the most significantly affected. This is in accord with the small number of articles written about the effect of dendrimers on other cell types (11,22). The number of selected regulated genes is greater after treatment with dendriplex than with dendrimer alone. Furthermore, most of the genes dendriplex increase the effect of 2G-NN16 alone. It has been described that in human A431 cells the complex of Polyfect (a PAMAM-based dendrimer) with a control siRNA increases the total number of gene expression changes (both up and down-regulated) (11).

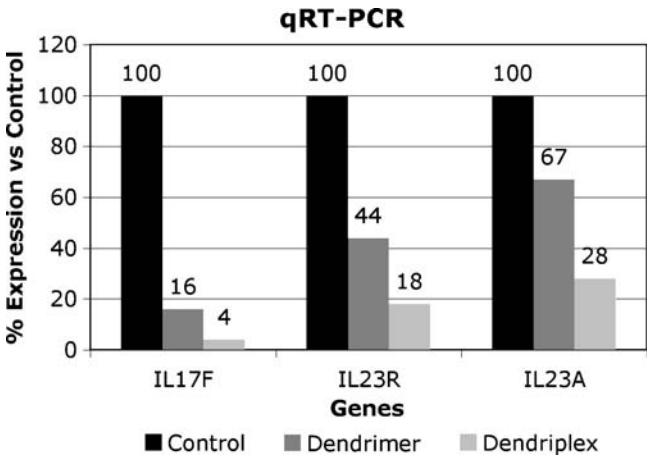


Fig. 5. Quantitative real-time RT-PCR of IL17F, IL23R and IL23A in macrophages exposed to 5 μM of dendrimer or dendriplex for 5 h. Un-treated macrophages were used as the control and are represented as 100% of the signal. A strong down-regulation is observed for all genes in response to dendrimer and dendriplex treatment.

One of the most interesting effects on macrophages by NN16 treatment is the strong down-regulation of Interleukin-17F (IL17F). Analysis using qRT-PCR demonstrates that ILF17 and the related IL23R and IL23A are severely repressed in macrophages in presence of 2G-NN16. IL17F belongs to the IL17 family and is only secreted by activated CD4 T cells and activated macrophages (23). Among the IL17 family, IL17F is most closely related to IL17 (24). The biological activities of IL17F are similar to those of IL17 and it regulates the production of IL-6, IL-8, G-CSF, and controls cartilage matrix turnover by increasing matrix release and inhibiting new matrix synthesis (24,25). IL17F also inhibits angiogenesis and induces production of IL2, TGF-ß and monocyte chemoattracting protein (MCP)-1 in endothelial cells (23). IL17 and IL17F form biologically active heterodimers with intermediate potency in inducing inflammatory genes (26,27). IL17, IL17F and its regulator IL23 appear to be important in host defense against extracellular bacteria and fungi (28–30). Additionally, a number of autoimmune diseases, such as psoriasis and inflammatory bowel disease, are associated with over-production of these cytokines and interference with IL17 production or action attenuates autoimmune disease (31–36). Polymorphisms in

the IL23R gene confer protection against psoriasis (37) and are associated with inflammatory bowel disease (38). Overexpression of IL-17A or IL-17F in the lung results in induction of CXC chemokines and neutrophil recruitment. In a case-control study of 1,125 unrelated Japanese subjects, a change in the third exon of the IL17F gene that causes a change of His161 to Arg161 (H161R) was shown to be associated with asthma and chronic obstructive pulmonary disease (COPD). Functionally, this variant failed to induce cytokines and chemokines and, interestingly, was able to antagonize the activity of wild-type IL-17F.

It is remarkable that many genes associated with the IL17 and IL23 pathway have been selected as differentially expressed genes in macrophages exposed to 2G-NN16, such as, CXCL1, CXCL2, IL-8, IL-1ß, Histone H3 and NF-κB. These results, together with those obtained by qRT-PCR showing a clear repression of IL17, IL23R and also IL23A, suggest that 2G-NN16 is down-regulating the IL23 pathway in macrophages. It remains to be seen if 2G-NN16 is also able to repress this pathway in CD4+ cells.

CONCLUSIONS

Analysis of global gene expression profiles in human primary macrophages in culture with 2G-NN16 dendrimer shows multiple changes, principally affecting immune system, proliferation and transcription regulation pathways. Our results illustrate the importance of performing this type of genomic analysis for all the non-viral delivery systems in order to know the effect exerted on the cell studied in each particular case. Dendrimer effects can overlap with the transported molecule effect. IL17F, the receptor for its regulator IL23R and the p19 subunit of the ligand IL23 are dramatically down-regulated in human macrophages by treatment with 2G-NN16 dendrimer or dendriplex. The repression of IL17F, IL23R and IL23A in macrophages by 2G-NN16 dendrimer makes this dendrimer a good candidate for the study for the treatment of autoimmune diseases.

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REFERENCES

1. S. D. Caruthers, S. A. Wickline, and G. M. Lanza. Nanotechnological applications in medicine. *Curr. Opin. Biotechnol.* **18**:26–30 (2007). doi:10.1016/j.copbio.2007.01.006.
2. P. S. Lai, P. J. Lou, C. L. Peng, C. L. Pai, W. N. Yen, M. Y. Huang, T. H. Young, and M. J. Shieh. Doxorubicin delivery by polyamidoamine dendrimer conjugation and photochemical internalization for cancer therapy. *J. Control. Release*. **122**:39–46 (2007). doi:10.1016/j.jconrel.2007.06.012.
3. H. Kang, R. DeLong, M. H. Fisher, and R. L. Juliano. Tat-conjugated PAMAM dendrimers as delivery agents for antisense and siRNA oligonucleotides. *Pharm. Res.* **22**:2099–2106 (2005). doi:10.1007/s11095-005-8330-5.
4. D. H. Kim, M. Longo, Y. Han, P. Lundberg, E. Cantin, and J. J. Rossi. Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat. Biotechnol.* **22**:321–325 (2004). doi:10.1038/nbt940.
5. T. M. Fahmy, J. P. Schneck, and W. M. Saltzman. A nanoscopic multivalent antigen-presenting carrier for sensitive detection and drug delivery to T cells. *Nanomedicine.* **3**:75–85 (2007).
6. J. F. Bermejo, P. Ortega, L. Chonco, R. Eritja, R. Samaniego, M. Mullner, E. de Jesus, F. J. de la Mata, J. C. Flores, R. Gomez, and A. Munoz-Fernandez. Water-soluble carbosilane dendrimers: synthesis biocompatibility and complexation with oligonucleotides; evaluation for medical applications. *Chem. Eur. J.* **13**:483–495 (2007). doi:10.1002/chem.200600594.
7. R. Duncan, and L. Izzo. Dendrimer biocompatibility and toxicity. *Adv. Drug Deliv. Rev.* **57**:2215–2237 (2005). doi:10.1016/j.addr.2005.09.019.
8. S. Kawakami, J. Wong, A. Sato, Y. Hattori, F. Yamashita, and M. Hashida. Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. *Biochim. Biophys. Acta.* **1524**:258–265 (2000).
9. J. H. Kuo, M. S. Jan, and Y. L. Lin. Interactions between U-937 human macrophages and poly(propyleneimine) dendrimers. *J. Control. Release.* **120**:51–59 (2007). doi:10.1016/j.jconrel.2007.03.019.
10. L. Chonco, J. F. Bermejo-Martin, P. Ortega, D. Shcharbin, E. Pedziwiatr, B. Klajnert, F. J. de la Mata, R. Eritja, R. Gomez, M. Bryszewska, and M. A. Munoz-Fernandez. Water-soluble carbosilane dendrimers protect phosphorothioate oligonucleotides from binding to serum proteins. *Org. Biomol. Chem.* **5**:1886–1893 (2007). doi:10.1039/b703989a.
11. A. J. Hollins, Y. Omidi, I. F. Benter, and S. Akhtar. Toxicogenomics of drug delivery systems: Exploiting delivery system-induced changes in target gene expression to enhance siRNA activity. *J. Drug Target.* **15**:83–88 (2007). doi:10.1080/10611860601151860.
12. R. Edgar, M. Domrachev, and A. E. Lash. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic. Acids Res.* **30**:207–210 (2002). doi:10.1093/nar/30.1.207.
13. D. Rubio, S. Garcia, M. F. Paz, T. De la Cueva, L. A. Lopez-Fernandez, A. C. Lloyd, J. Garcia-Castro, and A. Bernad. Molecular characterization of spontaneous mesenchymal stem cell transformation. *PLoS One*. **3**:e1398 (2008). doi:10.1371/journal.pone.0001398.
14. P. B. Dallas, N. G. Gottardo, M. J. Firth, A. H. Beesley, K. Hoffmann, P. A. Terry, J. R. Freitas, J. M. Boag, A. J. Cummings, and U. R. Kees. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR—how well do they correlate? *BMC Genomics.* **6**:59 (2005). doi:10.1186/1471-2164-6-59.
15. B. Oppmann, R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J. S. Abrams, K. W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J. F. Bazan, and R. A. Kastelein. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* **13**:715–725 (2000). doi:10.1016/S1074-7613(00)00070-4.
16. N. K. Jain, and A. Asthana. Dendritic systems in drug delivery applications. *Expert Opin. Drug Deliv.* **4**:495–512 (2007). doi:10.1517/17425247.4.5.495.
17. R. Rupp, S. L. Rosenthal, and L. R. Stanberry. VivaGel (SPL7013 Gel): a candidate dendrimer–microbicide for the prevention of HIV and HSV infection. *Int. J. Nanomedicine*. **2**:561–566 (2007).
18. F. Alexis, J. W. Rhee, J. P. Richie, A. F. Radovic-Moreno, R. Langer, and O. C. Farokhzad. New frontiers in nanotechnology for cancer treatment. *Urol. Oncol.* **26**:74–85 (2008). doi:10.1016/j.urolonc.2007.03.017.

19. L. Vannucci, A. Fiserova, K. Sadalapure, T. K. Lindhorst, M. Kuldova, P. Rossmann, O. Horvath, V. Kren, P. Krist, K. Bezouska, M. Luptovcova, F. Mosca, and M. Pospisil. Effects of N-acetyl-glucosamine-coated glycodendrimers as biological modulators in the B16F10 melanoma model *in vivo*. *Int. J. Oncol.* **23**:285–296 (2003).
20. S. Shaunak, S. Thomas, E. Gianasi, A. Godwin, E. Jones, I. Teo, K. Mireskandari, P. Luthert, R. Duncan, S. Patterson, P. Khaw, and S. Brocchini. Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation. *Nat. Biotechnol.* **22**:977–984 (2004). doi:10.1038/nbt995.
21. Y. Omidi, A. J. Hollins, R. M. Drayton, and S. Akhtar. Polypropylenimine dendrimer-induced gene expression changes: the effect of complexation with DNA, dendrimer generation and cell type. *J. Drug Target.* **13**:431–443 (2005). doi:10.1080/10611860500418881.
22. S. Akhtar, and I. Benter. Toxicogenomics of non-viral drug delivery systems for RNAi: potential impact on siRNA-mediated gene silencing activity and specificity. *Adv. Drug Deliv. Rev.* **59**:164–182 (2007). doi:10.1016/j.addr.2007.03.010.
23. T. Starnes, M. J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H. E. Broxmeyer, and R. Hromas. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* **167**:4137–4140 (2001).
24. S. D. Hurst, T. Muchamuel, D. M. Gorman, J. M. Gilbert, T. Clifford, S. Kwan, S. Menon, B. Seymour, C. Jackson, T. T. Kung, J. K. Brieland, S. M. Zurawski, R. W. Chapman, G. Zurawski, and R. L. Coffman. New IL-17 family members promote Th1 or Th2 responses in the lung: *in vivo* function of the novel cytokine IL-25. *J. Immunol.* **169**:443–453 (2002).
25. S. G. Hymowitz, E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, G. Pan, A. L. Gurney, A. M. de Vos, and M. A. Starovasnik. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* **20**:5332–5341 (2001). doi:10.1093/emboj/20.19.5332.
26. S. H. Chang, and C. Dong. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res.* **17**:435–440 (2007).
27. J. F. Wright, Y. Guo, A. Quazi, D. P. Luxenberg, F. Bennett, J. F. Ross, Y. Qiu, M. J. Whitters, K. N. Tomkinson, K. Dunussi-Joannopoulos, B. M. Carreno, M. Collins, and N. M. Wolfman. Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. *J. Biol. Chem.* **282**:13447–13455 (2007). doi:10.1074/jbc.M700499200.
28. K. I. Happel, P. J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L. J. Quinton, A. R. Odden, J. E. Shellito, G. J. Bagby, S. Nelson, and J. K. Kolls. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. *J. Exp. Med.* **202**:761–769 (2005). doi:10.1084/jem.20050193.
29. E. V. Acosta-Rodriguez, L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* **8**:639–646 (2007). doi:10.1038/ni1467.
30. S. LeibundGut-Landmann, O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland, and C. Reis e Sousa. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* **8**:630–638 (2007). doi:10.1038/ni1460.
31. C. Lock, G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, P. Klonowski, A. Austin, N. Lad, N. Kaminski, S. J. Galli, J. R. Oksenberg, C. S. Raine, R. Heller, and L. Steinman. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat. Med.* **8**:500–508 (2002). doi:10.1038/nm0502–500.
32. A. Vaknin-Dembinsky, K. Balashov, and H. L. Weiner. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J. Immunol.* **176**:7768–7774 (2006).
33. S. Fujino, A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut.* **52**:65–70 (2003). doi:10.1136/gut.52.1.65.
34. S. Kotake, N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M. T. Gillespie, T. J. Martin, and T. Suda. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* **103**:1345–1352 (1999). doi:10.1172/JCI5703.
35. M. I. Koenders, L. A. Joosten, and W. B. van den Berg. Potential new targets in arthritis therapy: interleukin (IL)-17 and its relation to tumour necrosis factor and IL-1 in experimental arthritis. *Ann. Rheum. Dis.* **65**(Suppl 3):iii29–iii33 (2006). doi:10.1136/ard.2006.058529.
36. M. B. Teunissen, C. W. Koomen, R. de Waal Malefyt, E. A. Wierenga, and J. D. Bos. Interleukin-17 and interferon-gamma synergize in the enhancement of proinflammatory cytokine production by human keratinocytes. *J. Invest. Dermatol.* **111**:645–649 (1998). doi:10.1046/j.1523-1747.1998.00347.x.
37. F. Capon, P. Di Meglio, J. Szaub, N. J. Prescott, C. Dunster, L. Baumber, K. Timms, A. Gutin, V. Abkevic, A. D. Burden, J. Lanchbury, J. N. Barker, R. C. Trembath, and F. O. Nestle. Sequence variants in the genes for the interleukin-23 receptor (IL23R) and its ligand (IL12B) confer protection against psoriasis. *Hum. Genet.* **122**:201–206 (2007). doi:10.1007/s00439-007-0397-0.
38. D. McGovern, and F. Powrie. The IL23 axis plays a key role in the pathogenesis of IBD. **Gut**. **56**:1333–1336 (2007). doi:10.1136/gut.2006.115402.