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Carbosilane Dendrimer 2G-NN16 Represses Tc17 Differentiation in Primary T CD8+ Lymphocytes

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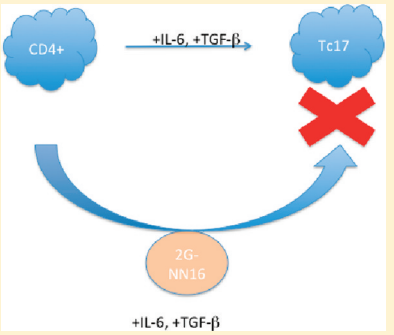
Supporting Information

ABSTRACT: We studied changes in gene expression induced by the carbosilane dendrimer 2G-NN16 to evaluate their potential as a vehicle for gene therapy and as medication. Global gene expression profiles on CD8+ T lymphocytes reveal that ribosomal proteins are induced in the presence of 2G-NN16. IL17A and IL17F, the principal interleukins secreted by Tc17 cells, a subset of CD8+ T lymphocytes, were down-regulated when cultured in the presence of this dendrimer. Microarray results were confirmed by real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). 2G-NN16 also showed a high potential for in vitro inhibition of Tc17 differentiation of CD8+ T lymphocytes in the presence of the Tc17 differentiation molecules IL6 and TGF-B1. These findings suggest that 2G-NN16 could facilitate drug delivery and may be used to treat inflammatory processes driven by Tc17 cells.



KEYWORDS: *Tc17 cells, dendrimers, nanoparticles, gene expression, immune response*

INTRODUCTION

Nanomedicine is an emerging field in which promising advances have been made in the past decade. Nanocompounds have been widely applied in biomedicine in several different ways.[1] Dendrimers are repeatedly branched, roughly spherical large nanocompounds that have proven useful in drug delivery applications.[2] Toxicity studies are usually performed for dendrimers to ensure the viability of the cells. However, knowledge of the changes in gene expression induced by these compounds is essential before they can be considered for gene delivery or other applications.[3] In this sense, microarray profiling has revealed nonspecific changes in gene expression in human lung cancer A549 cells upon treatment with polyamidoamine (PAMAM) dendrimers alone or when complexed with an anti-EGFR (epidermal growth factor receptor) antisense oligonucleotide.[4] Treatment with polypropilenimine diaminobutane dendrimers showed up-regulation of the EGFR and Akt kinase genes in A549 cells but not in A431 cells, suggesting that gene expression changes induced by dendrimers are dependent on cell type.[5] Moreover, these changes have revealed a potential therapeutic function for these molecules.

In drug delivery, the principal advantage of nonviral vectors against viral vectors is that the former are more versatile and induce a less intense immune response.[6] However, the mannosylated PAMAM dendrimer ovoalbumin (OVA) has been reported to be a potent inducer of the immune response, generating a strong OVA-specific CD4+ and CD8+ T-cell response.[7] CD8+ T lymphocytes play a central role in the immune response. When these cells are activated and differentiated, they become cytotoxic and induce the death of infected cells.[8] Activation of the immune system could prove advantageous when we investigate mechanisms to boost cell death in diseases such as cancer. However, reduction of immune activation has been suggested to be beneficial for certain groups of HIV-infected individuals.[9] For this reason, detailed characterization of processes altered in the target cell by the drug delivery agent is crucial if therapy is to be successful.

2G-NN16 is a second-generation dendrimer with two peripheral nitrogen atoms per branch and 16 positive charges. It has a carbosilane skeleton and a cationic surface consisting of 16 uniformly distributed quaternized amines and can bind and release small nucleic acids such as oligonucleotides, siRNAs, and peptides.[10] In antiretroviral therapy, 2G-NN16 has proven to be an efficient vehicle for siRNA delivery, by crossing the blood−brain barrier and increasing functionality compared with siRNA alone.[11] When injected, the first contact for most dendrimers is with cells of the immune system. Analysis of the gene expression profile of human primary monocytes treated with 2G-NN16 revealed that immune response genes, mainly cytokines involved in the Th17 response—IL17F, IL23A, and IL23R—were dysregulated.[12] Two cell populations of CD4+ T and CD8+ T lymphocytes—Th17 and Tc17, respectively—are the main producers of these cytokines.

Tc17 cells have recently been reported to share most of the interleukin secreted with Th17 cells.[13] These cells are

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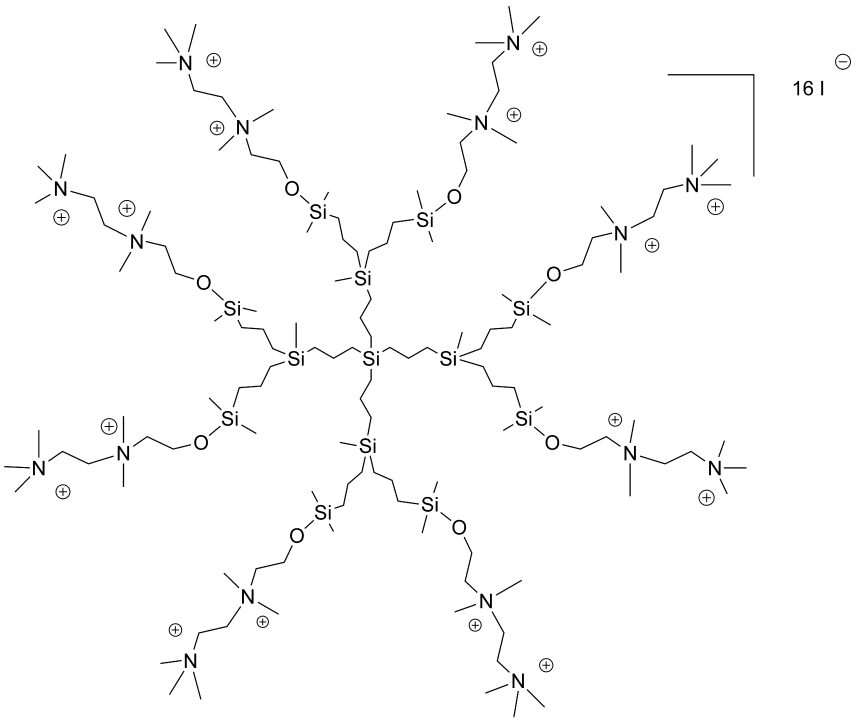

Figure 1. Complete chemical structure of the dendrimer 2G-NN16.

characterized by high expression of IL17A and IL17F interleukins. Tc17 cells have recently been phenotypically characterized.[14] This characterization indicates that Tc17 lymphocytes express a unique phenotype and chemokin receptors and they differentiate from the same precursors that differentiate into IFN-g-producing CD8+ T lymphocytes. These findings suggest that Tc17 cells are a unique cell lineage in terms of both function and differentiation. However, the role of this cell population is not clear. A role for Tc17 cells in regulating disease progression during pathogenic simian immunodeficiency virus (SIV) infection has been suggested, because a lower Tc17 count was observed in SIV-infected macaques with AIDS than in healthy or antiretroviral-treated macaques.[15] A role in acute graft-versus-host disease in patients undergoing unmanipulated blood and marrow transplantation has been described in Th17 and Tc17 cells.[16] Remarkably, a significant proportional rise in Tc17 and Tc22 cells but not in Th17 and Th22 cells was found in T cells isolated from psoriatic versus normal skin[17] or systemic lupus erythematosus.[18] Having a compound that allows us to regulate the differentiation of Tc17 cells can help us to treat this disease and new ones to be discovered in the future.

We used microarray technology to study changes in gene expression induced in CD8+ T lymphocytes by 2G-NN16 to evaluate their effect on IL17-related interleukins and Tc17 differentiation. We analyzed the biological functions altered and assessed the viability of this dendrimer as a drug delivery vehicle in biomedical applications.

MATERIALS AND METHODS

Synthesis of 2G-NN16. 2G-$[Si\{O(CH_2)_2N^+(Me)_2(CH_2)_2N^+(Me)_3(I^-)_2\}]_8$, a carbosilane dendrimer, was used in the experiments. This dendrimer was synthesized as previously described and obtained as a white water-soluble solid.[10] 2G-NN16 contains 8 Si−O bonds that give it the ability to degrade over time in water. The hydrolysis of the carbosilane dendrimer used in this study is a slow process, taking 12−24 h after being dissolved in water. 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 the dendrimer was not degraded. The structure of this dendrimer is shown in Figure 1. 2G-NN16 was dissolved in phosphate buffered saline (PBS) and immediately added to cell cultures.

Isolation and Culture of CD8+ T Cells. Some of the peripheral blood mononuclear cell (PBMC) samples used in this study were kindly provided by the Spanish HIV HGM BioBank integrated in the Spanish AIDS Research Network (RIS)[19] Peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals and processed by a Ficoll density gradient centrifugation. CD8+ T lymphocytes were negatively selected from PBMCs using the Pan T Cell Isolation Kit followed by positive selection with anti-CD8 (Miltenyi Biotec, Bergisch Gladbach, Germany).

For microarrays, before isolation of CD8+ T cells, PBMCs were activated with 60 U/mL of IL2 and 2 μg/mL of phytohemagglutinin for 3 days in RPMI medium enriched with 10% fetal bovine serum and 1% ampicillin, 1% cloxacillin, 0.32% gentamicin, and 2 nM glutamine at 37 °C in a 5% CO_2 atmosphere. CD8+ T lymphocytes were then grown to 1×10^6 per well over 5 h in RPMI-enriched medium and 5 μM 2G-NN16 or PBS. Finally, cells were centrifuged and the supernatant removed. Cells were stored at −80 °C until RNA extraction.

For Tc17 differentiation, purified CD8+ T lymphocytes at a density of 1×10^6 cells/well were cultured for 3 days in RPMI medium enriched with 10% fetal bovine serum, 1% ampicillin,

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1% cloxacillin, 0.32% gentamicin, 2 nM glutamine, 2 μg/mL α-CD3, 0.5 μg/mL α-CD28, 50 ng/mL IL6, or 10 ng/mL TGF-β1 and in the presence of 5 μM 2G-NN16 every 24 h or PBS 1X at 37 °C in a 5% CO_2 atmosphere. CD8+ T lymphocytes were cultured in the absence of IL6 and TGF-β1 as a negative control of Tc17 differentiation.

Cytotoxicity Assays. Toxicity assays were performed from four different donors by measuring lactate dehydrogenase (LDH) and propidium iodide. CD8+ T lymphocytes were grown at a concentration of 1×10^6 cells/well and exposed to 5 μM 2G-NN16 or PBS in RPMI medium enriched with 10% fetal bovine serum and 1% ampicillin, 1% cloxacillin, 0.32% gentamicin, and 2 nM glutamine at 37 °C in a 5% CO_2 atmosphere. The medium was changed every 24 h for 5 days with a fresh aliquot of 2G-NN16. Cells were then rinsed in PBS and centrifuged at 2500 rpm for 10 min. Cells were analyzed to assess cell death using propidium iodide and supernatant to measure cell toxicity using the LDH assay. LDH cytotoxicity was measured using the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Fitchburg, WI) following the manufacturer's instructions. The absorbance was read using the Anthos 2001 microplate reader (Innogenetics NV, Gent, Belgium). CD8+ T cells exposed to either 2G-NN16 or PBS were incubated in the dark with 5 μL of propidium iodide (1 mg/mL) solution for 30 min at room temperature and analyzed in an FC500 flow cytometer (Beckman Coulter, Brea, CA) to determine the number of dead CD8+ T cells.

RNA Extraction. CD8+ T cells were purified and exposed to 5 μM dendrimer or PBS as described above. RNA from CD8+ T cells (approximately 1×10^6 cells/well for each condition) from three donors was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions. RNA concentrations were measured using the Nanodrop ND-100 UV−vis spectrophotometer (Thermo Scientific, Wilminton, DE). When necessary, RNA was concentrated using a 5301 concentrator (Eppendorf, Hamburg, Germany). Before carrying out the microarray chip assays, RNA integrity was verified using the Agilent RNA 6000 Nano Kit in a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarrays. Microarray experiments were performed following the MIAME (Minimal Information About a Microarray Experiment) criteria. For each sample, 1 μg of total RNA was amplified and labeled using the SuperScript Indirect RNA Amplification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, RNA was denatured and reverse transcribed to cDNA. This cDNA was purified and transcribed in vitro to RNA using T7 RNA polymerase. Amplified RNA was purified and quantified using a Nanodrop ND-100 UV−vis spectrophotometer. Control and dendrimer probes were labeled, respectively, with an Alexa Fluor 555 reactive dye decapack and Alexa Fluor 647 reactive dye decapack. Labeled RNA was again purified and quantified using a Nanodrop ND-100 UV−vis spectrophotometer, and the RNA quality was checked using the Agilent 2100 bioanalyzer. Agilent 4X44 K whole genome human chips (Agilent Technologies) were hybridized, washed, and scanned as described elsewhere.[12] This microarray contains probes for 41 000 unique genes and transcripts that have been verified and optimized by the manufacturer.

Raw microarray data were loaded into the MIAME-compliant Almazen database software (http://almazen.bioalma.com). The background was subtracted and each experiment normalized by total intensity and subgrid LOWESS (locally weighted scatterplot smoothing). A gene was considered to be induced or repressed when all of the following criteria were fulfilled: ratio NN16/control > 2 or < −2, p value of <0.05, z scores of > 2 or < −2, SD log-ratio < 0.6, and an average signal of > 32 (to exclude spots close to background).

Data Analysis. Potential signaling pathways and functions related to selected genes were analyzed using ingenuity pathway analysis (IPA) (http://www.ingenuity.com). This application reveals relevant networks by comparing the gene set with known signaling pathways. It then assigns 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% confidence level). Biological functions are then calculated and assigned to each network.

Gene set enrichment analysis (GSEA) of differentially expressed CD8+ genes after exposure to 2G-NN16 was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 bioinformatics resources (http://david.abcc.ncifcrf.gov).[20] It consists of an integrated biological knowledge database and analytical tools aimed at systematically extracting biological significance from a large gene/protein list. Gene Ontology-enriched terms or enriched functional-related gene groups help to interpret complex gene lists from microarray experiments. An enrichment score of >1.5 was considered significant.

Real-Time qRT-PCR. Total RNAs from CD8+ T cells cultured in the presence or absence of 2G-NN16 dendrimer were isolated from four different donors as previously described. cDNA was generated from 200 ng of total RNA using the high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a 10 μL final reaction volume following the manufacturer's instructions. Real-time PCR was performed in triplicate using 2 μL/well of a dilution performed for 1/10 of each cDNA, 0.04 μM CXCR4, IL17F, IL23R, IL23A, or IL17A (CXCR4 forward, 5′-GGC CGA CCT CCT CTT TGT C-3′; CXCR4 reverse, 5′-TTG CCA CGG CAT CAA CTG-3′; IL17F forward, 5′-GGC ATC ATC AAT GAA AAC CA-3′; IL17F reverse, 5′-TGG GGTCCC 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′; IL17A forward, 5′-TGG GAA GAC CTC ATT GGT GT-3′; IL17A reverse, 5′-GGA TTT CGT GGG ATT GTG AT-3′), 1× SYBR Green PCR Master Mix in 25 μL of total volume reaction in MicroAmp Fast 96-well reaction plate (0.1 mL) (Applied Biosystems, Foster City, CA). PCR reactions were run on an Applied Biosystems StepOne Plus Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Foster City, CA). CXCR4 was used as a normalization gene. The results were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) in StepOne Software v2.1 (Applied Biosystems, Foster City, CA) with a confidence level (bar errors) of 95%. Ct values of CD8+ lymphocytes not exposed to 2G-NN16 dendrimer were used as a control for relative quantification.

Enzyme-Linked Immunosorbent Assay (ELISA). IL17F and IL17A were measured in the supernatant of CD8+ T lymphocytes cultured for 3 days in Tc17 differentiating conditions (see Isolation and Culture of T CD8+ Cells)

using an IL17A ELI-Pair for ELISA or IL17F ELI-Pair for ELISA (Gen-Probe, San Diego, CA) following the manufacturer's instructions.

RESULTS

2G-NN16 Toxicity in CD8+ Cells. The toxicity was measured using the propidium iodide and LDH assays in purified CD8+ T cells by exposure to 5 μM 2G-NN16 every 24 h for 5 days (Figure 2). This concentration was selected based on siRNA inhibition experiments and previous toxicity data obtained with other cell types.[11,21] The dendrimer at 5 μM produced cell mortality of 5.83%, as determined by the propidium iodide assay. Analysis of these same conditions using the LDH assay revealed no significant cell mortality. Given that compounds inducing a mortality rate higher than 10% are considered toxic, we can conclude that 2G-NN16 at 5 μM does not produce a significant effect on viability in CD8+ T lymphocytes after 5 days' incubation.

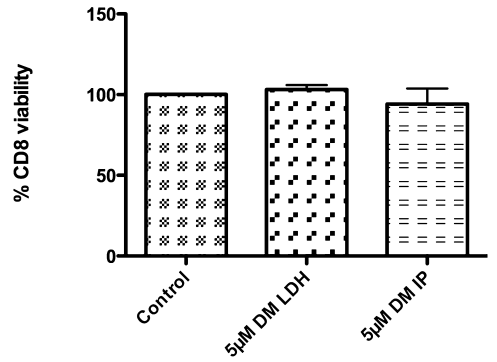

Figure 2. Toxicity of 2G-NN16 on CD8+ T lymphocytes. Percentage of viability of CD8+ T cells after exposure to 5 μM 2G-NN16 measured by lactate dehydrogenase (LDH) or propidium iodide (PI). A control of 100% is used to normalize the data.

Differentially Expressed Genes in CD8+ T Cells after Exposure to 2G-NN16. Transcriptome profiles were studied in CD8+ T cells exposed to 5 μM of 2G-NN16 for 5 h. A comparison against the same cells without exposure to the dendrimer using the criteria defined in the methods section revealed 355 different genes whose expression level changed (325 were up-regulated and 30 down-regulated; Table 1 of the Supporting Information). The most repressed known genes were deleted in azoospermia-like (DAZL), fucosyltransferase 7 (FUT7), G protein-coupled receptor 15 (GPR15) (3.6-fold repressed, 3.2-fold repressed, and 2.78-fold repressed, respectively) and 2 interleukins, interleukin 21 (IL21) and interleukin 23 receptor (IL23R) (2.7-fold and 2.64-fold repressed, respectively). As for the known genes most induced by dendrimer exposure, we identified contactin-associated protein-like 3 (CNTNAP3), ZCRB1, signal recognition particle 9 kDa (SRP9), and SMT3 suppressor of mif two 3 homologue 2 (SUMO2) (4.3-fold, 4.1-fold, 3.9-fold, and 3.9-fold induced, respectively). Interestingly, up to 24 ribosomal protein coding genes were up-regulated in CD8+ T cells exposed to 2G-NN16. A summary of the top 50 most differentially expressed genes is shown in Table 1 (25 induced and 25 repressed).

Functional Analysis. Functional data on the 355 genes that were differentially expressed in CD8+ T cells after exposure to dendrimer were extracted using IPA and the DAVID database. IPA offers a view of the most likely represented functions, interactions, and pathways. Table 2 shows the functions with the highest probability of being represented in the selected genes. The principal functions were protein synthesis (p value from 6.08×10^{-9} to 9.25×10^{-9}) and the metabolism of protein (p value of 1.16×10^{-5}).

A graphic of the most significant network identified is shown in Figure 3. The principal functions represented in this network are DNA replication, recombination, and repair, as well as RNA post-transcriptional regulation and gene expression.

Gene annotation enrichment analysis was performed using DAVID v6.5. One gene cluster was identified with an enrichment score of 12.02 (equivalent to a mean p value of 10^{-12}) including the following Gene Ontology terms: cytosolic ribosome, ribosomal subunit, ribonucleoprotein complex, ribosome, cytosolic part, cytosolic large ribosomal subunit, large ribosomal subunit, cytosol, cytosolic small ribosomal subunit, small ribosomal subunit, and transcriptional elongation and translation. These results confirm that protein synthesis and related functions are the most affected pathways in CD8+ T lymphocytes exposed to 2G-NN16.

Microarray Validation. A real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with two objectives: first, to validate microarray data and, second, to study the effect of 2G-NN16 on key molecules in the Tc17 response (Figure 4). For this purpose, four genes were selected: IL23R, IL23A, IL17F, and IL17A. IL23R was 1.89-fold repressed in CD8+ T cells exposed to 2G-NN16. This result is similar to that of the microarray approach, where IL23R was 2.64-fold repressed. Real time qRT-PCR is a more sensitive technique than microarray; consequently, the extent of the gene changes are not the same as those observed in microarray. Real-time qRT-PCR results for IL23A expression revealed no regulation (1.22-fold repressed), as was the case in microarray (1.17-fold repressed). IL17F was 3.44-fold repressed in CD8+ T cells exposed to 2G-NN16. This reduction was similar to that found in the microarray experiments (3.49-fold repressed). However, this gene did not pass the selection filter, because, in the first case, the SD log ratio was slightly higher than required (0.79 when the limit was set at 0.70) and, in the second, the p value was greater than 0.05 (p value = 0.058, when the limit was set at 0.05). Finally, real-time qRT-PCR revealed down-regulation of IL17A (2.22-fold repressed) in CD8+ T cells exposed to 2G-NN16. Again, this result is very similar to the 1.95-fold reduction observed in microarrays. These results confirm the data obtained by microarray and show that IL23R, IL17A, and IL17F are down-regulated in CD8+ T cells after exposure to 2G-NN16.

Effect of 2G-NN16 on Tc17 Differentiation. To study the effects of 2G-NN16 on Tc17 differentiation, CD8+ T lymphocytes from three donors were cultured in Tc17 differentiating conditions (IL6 and TGF-β1) and exposed to 5 μM 2G-NN16 or PBS for 3 days. After this period, IL17A and IL17F were measured using ELISA to evaluate Tc17 differentiation (Figure 5). A clear reduction in IL17A and IL17F for all samples was observed, indicating a loss of Tc17 differentiation capability of CD8+ T lymphocytes in the presence of 2G-NN16.

DISCUSSION

Gene expression changes in CD8+ T lymphocytes after exposure to 2G-NN16 were identified and analyzed to study both the viability of this dendrimer as a drug delivery vehicle in biomedical applications and the effect on Tc17 response. CD8+

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Table 1. Fifty Most Differentially Expressed Genes in CD8+ T Lymphocytes after Exposure to 2G-NN16[a]

symbol	description	fold change
DAZL	deleted in azoospermia-like	−3.58
FUT7	fucosyltransferase 7 (α-(1,3)-fucosyltransferase)	−3.20
GPR15	G protein-coupled receptor 15	−2.78
A_24_P170309		−2.73
IL21	interleukin 21	−2.71
IL23R	interleukin 23 receptor	−2.65
ABCG2	ATP-binding cassette, subfamily G member 2	−2.63
C3AR1	complement component 3a receptor 1	−2.53
CA414006		−2.42
CCR4	chemokine (C−C motif) receptor 4	−2.39
A_23_P414771		−2.35
IL1R1	interleukin 1 receptor, type I	−2.33
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	−2.32
HSPA8	heat shock 70 kDa protein 8	−2.31
MBOAT7	membrane bound *O*-acyltransferase domain containing 7	−2.31
C6orf120	chromosome 6 open reading frame 120	−2.24
CTSH	cathepsin H	−2.24
CXCR6	chemokine (C-X-C motif) receptor 6	−2.18
AI207522		−2.17
FAR2	fatty acyl CoA reductase 2	−2.14
TRMT61A	tRNA methyltransferase 61 homologue A (*S. cerevisiae*)	−2.14
MBTPS2	membrane-bound transcription factor peptidase, site 2	−2.13
NELF	nasal embryonic LHRH factor	−2.13
ALKBH8	alkB, alkylation repair homologue 8 (*E. coli*)	−2.11
BMPR1A	bone morphogenetic protein receptor, type IA	−2.11
CASC5	cancer susceptibility candidate 5	3.32
MED13L	mediator complex subunit 13-like	3.38
RPS12	ribosomal protein S12	3.41
PDCD5	programmed cell death 5	3.43
LOC401863		3.45
PCMTD1	protein-L-isoaspartate (D-aspartate) *O*-methyltransferase domain containing 1	3.47
DUT	deoxyuridine triphosphatase	3.48
RPL21	ribosomal protein L21	3.51
BC065737	ribosomal protein S3A pseudogene	3.56
A_24_P75399		3.64
RPL14	ribosomal protein L14	3.65
A_24_P850187		3.67
A_24_P358337		3.74
LOC390411	nucleophosmin pseudogene	3.75
FABP5	fatty acid binding protein 5 (psoriasis-associated)	3.80
LOC402175	hypothetical gene supported by AF044957; NM_004547	3.81
LOC641844		3.83
A_24_P298099		3.86
SUMO2	SMT3 suppressor of mif two 3 homologue 2 (*S. cerevisiae*)	3.90
A_24_P170103		3.90
SRP9	signal recognition particle 9 kDa	3.92
ZCRB1	zinc finger CCHC-type and RNA binding motif 1	4.15
A_24_P93452		4.29
CNTNAP3	contactin associated protein-like 3	4.32
THC2637707		5.18

[a]A list containing the 25 most induced and 25 most repressed genes in CD8+ T lymphocytes. Gene symbol, a brief description, and fold change dendrimer/no-dendrimer are represented. When the gene symbol is not available, Agilent ID, Ensemble ID, TIGR ID, or GenBank are provided.

Table 2. Top Five Functions Associated with Regulated Genes in CD8+ T Lymphocytes Cultured in the Presence of 2G-NN16 Dendrimer Using Ingenuity Pathway Analysis

category	function annotation	*p*-value	molecules
protein synthesis	synthesis of protein	6.08×10^{-9} to 9.25×10^{-9}	CDKN1B, EIF1B, NPM1 (includes EG:4869), RPL14, RPL26, RPL30, RPL31, RPL34, RPL21 (includes EG:6144), EEF1A1, IL21, PHLDA1, RPL5, RPL6, RPL17, RPL24, RPL27A, RPL9 (includes EG:6133), RPS10, RPS12 (includes EG:6206), RPS3A
protein synthesis	metabolism of protein	0.0000116	CASP2, CDC23 (includes EG:8697), CTSH, EEF1A1, EIF1B, IL21, PHLDA1, RPL5, RPL6, RPL14, RPL17, RPL24, RPL26, RPL30, RPL31, RPL34, RPL21 (includes EG:6144), RPL27A, RPL9 (includes EG:6133), RPS10, RPS12 (includes EG:6206), RPS3A
cell cycle	delay in cell cycle progression of lymphoblastoid cell lines	0.000029	BCL2, CASP2, PCNA
cellular assembly and organization	biogenesis of protein-RNA complex	0.0000798	RPL5, RPL14, RPL26
cell cycle	S phase of lymphoma cell lines	0.000131	BCL2, CDKN1B

T-cell viability was not affected by exposure to 5 μM 2G-NN16. This concentration has been successfully used for this dendrimer in drug delivery or as a transfection reagent with low or insignificant toxicity in a variety of primary human cells (PBMCs, macrophages, or dendritic cells) and immortalized human cells (astrocytes and trophoblasts).[21]

Microarray analysis showed that 355 genes were up-regulated or down-regulated in CD8+ T cells after exposure to 2G-NN16. Functional annotation analysis revealed that Gene Ontology terms associated with "ribosome" were over-represented in the regulated genes. In fact, 24 ribosomal proteins were overexpressed after exposure to 2G-NN16 in CD8+ T lymphocytes. Ribosomes are the central part of the protein-synthesizing mechanism of the cell. They consist of two dissociable subunits that are produced in the nucleolus and exported from the nucleus to the cytoplasm, where they are assembled into functional ribosomes.[22] Ribosome biogenesis is a highly complex and energy-expending process that requires the interaction of more than 200 proteins and RNA molecules, including RNA polymerases (Pol I, II, and III), ribosomal proteins, rRNAs, and

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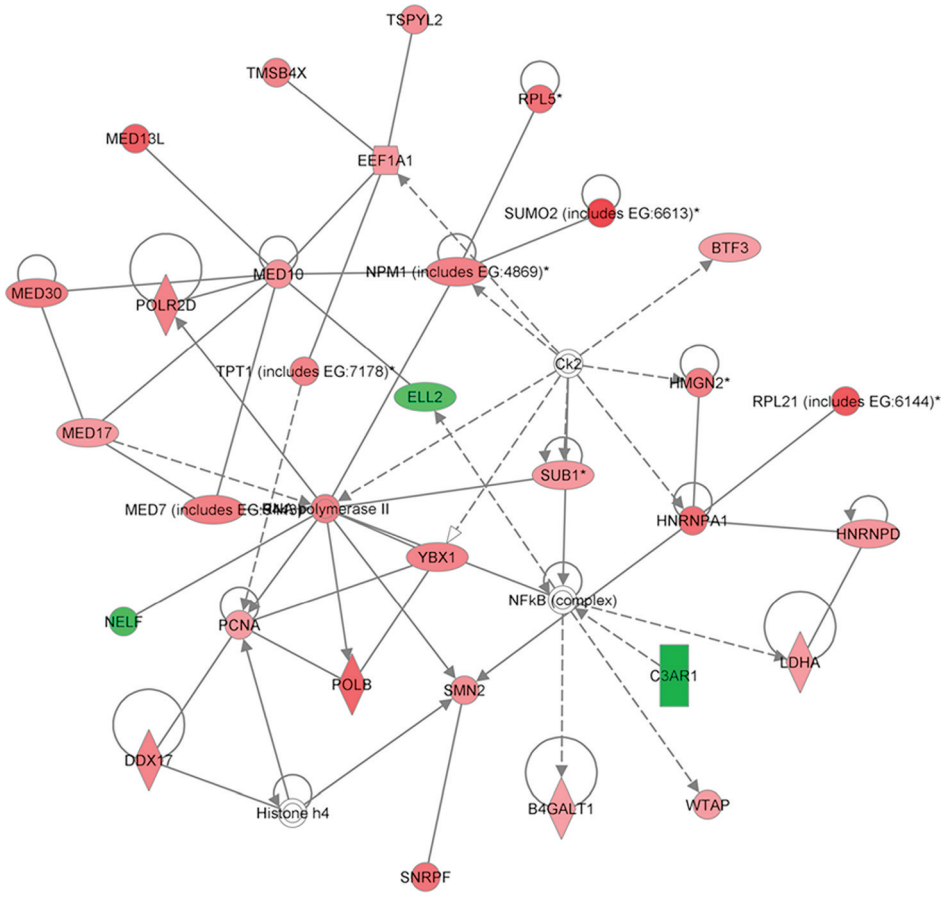

Figure 3. Graphical representation of the most significant interaction network of genes regulated by 2G-NN16 in CD8+ T lymphocytes. Regulated genes were uploaded to Ingenuity software, and gene interaction networks were created based on curated bibliography data. The red color represents overexpression, and green shows under-expression in CD8+ T lymphocytes exposed to 2G-NN16 dendrimer versus nonexposed.

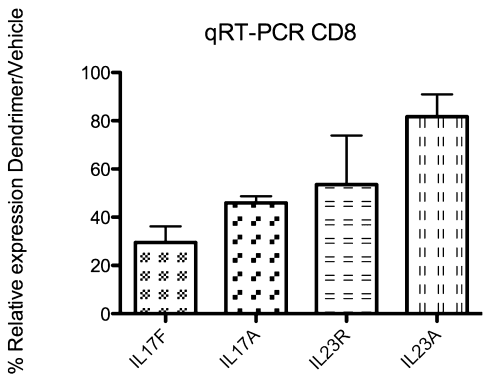

Figure 4. Real-time qRT-PCR on Tc17 cytokines. IL17F, IL17A, IL23A, and IL23R were quantified using real-time qPCR with the ΔΔCt method on total RNA from CD8+ T lymphocytes in the presence or absence of 2G-NN16. Samples were normalized using CXCR4. RNA from unexposed cells was used as a control for relative quantification (100%).

small molecules.[23] On the other hand, it is well-known that viruses activate translation machinery to produce viral proteins.[24] However, the lack of a ribosomal protein can doom a nascent subunit, leading potentially to accumulation of the other ribosomal proteins of the subunit. Moreover, autoregulation of ribosome synthesis can occur by transcription of rRNA in bacteria[25] and in eukaryotes.[26] Therefore, while up-regulation of ribosomal genes does not necessarily imply a functional increase in translation, this observation needs to be taken into consideration before promoting the use of 2G-NN16 in biomedical applications. Furthermore, we found a decrease in IL21 expression in CD8+ T lymphocytes exposed to 2G-NN16. IL21 was recently shown to play a pivotal role in controlling viral infections, as Il-21−/− and Il-21r−/− mice infected with choriomeningitis virus failed to contain the infection compared with wild-type animals.[27] One would expect that functions related to genes in interaction network 1 (Figure 3) overlap with top functions of the 355 genes differentially expressed in CD8+ T cells cultured with a dendrimer. However, ribosomal proteins are disseminated over all networks found for Ingenuity software, which dissipates the possibility of revealing "protein synthesis" as a significant function in network 1.

Other results support using 2G-NN16 in infectious diseases. Expression of CXCR6 and GPR15 decreased in CD8+ T cells after exposure to 2G-NN16. Both these molecules and CCR5 have been described as major coreceptors of HIV-2 variants isolated from individuals with and without plasma viremia.[28] This lower expression could help to reduce the infectivity of HIV-2 in CD8+ cells and support 2G-NN16 as a good candidate vehicle for gene delivery in antiretroviral applications. All of these data suggest that 2G-NN16 could have advantages and disadvantages as a drug delivery vehicle in infectious diseases, particularly with regard to CD8+ T lymphocytes.

Results were also analyzed to investigate new biomedical applications for 2G-NN16. IL21, IL1R1, CXCR6, and IL23R were significantly down-regulated in CD8+ T cells in the

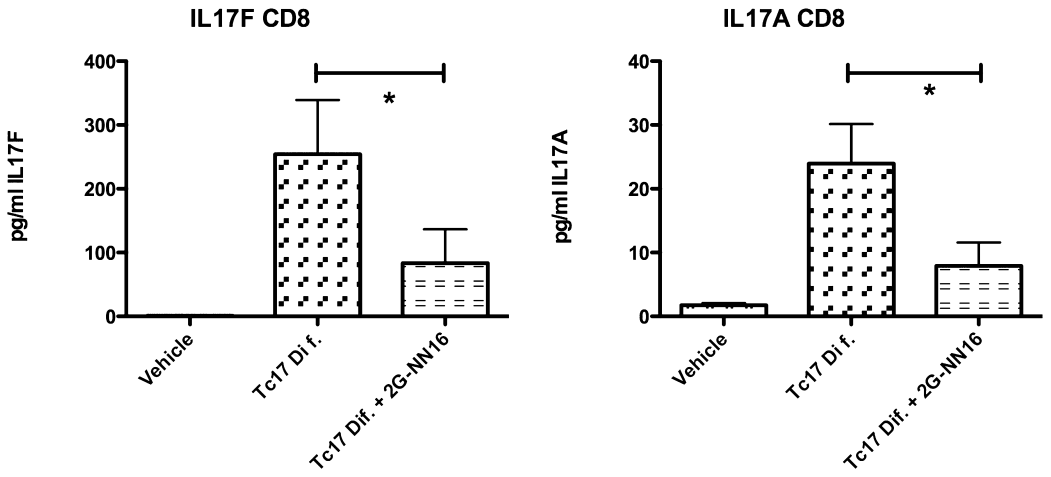

Figure 5. Effect of 2G-NN16 on IL17A and IL17F production in CD8+ T lymphocytes under Tc17 differentiating conditions. Protein levels of IL17A and IL17F were measured using ELISA in CD8+ T cells cultured in enriched RPMI medium, 2 μg/mL α-CD3, 0.5 μg/mL α-CD28, 50 ng/mL IL6, 10 ng/mL TGF-β1, and either 5 μM 2G-NN16 every 24 h (Tc17dif.+2G-NN16) or PBS 1X (Tc17 dif.) at 37 °C in a 5% CO_2 atmosphere. CD8+ T lymphocytes were cultured in RPMI-enriched medium as a negative control (nonstimulated).

presence of 2G-NN16. They are all expressed in Th17 and Tc17. Production of these genes has been related to autoimmune diseases, liver injury in patients infected with hepatitis B virus,[29] or myocardial inflammation.[30] A detailed analysis of microarray data on IL17A and IL17F showed a down-regulation on both transcripts when CD8+ cells were cultured in the presence of 2G-NN16 (1.96-fold and 3.5-fold, respectively). However, these genes did not pass the filtering conditions, which were highly restrictive. Our group had previously demonstrated that 2G-NN16 significantly reduces mRNA expression of IL17F, IL23R, and IL23A in human primary monocytes.[12] Recently, we have also observed how 2G-NN16 dendrimer repress Th17 response *in vitro* and *in vivo* (manuscript in preparation). All of these data suggest that this dendrimer has a strong capacity to repress genes involved in IL17 production in those cell types that participate in it. A comparison of the effect of dendrimer generation on IL17 repression level would be interesting. Unfortunately, 1G-NN8 is quickly degradated, and 3G-NN32 is not soluble in water, so it cannot be compared with the second generation.

Another question is how CD8+ T cells internalize dendrimer 2G-NN16, given that these cells are not phagocytic. Nanoparticles can be internalized by nonphagocytic pathways, such as pinocytosis, which can be conducted by almost every cell type.[31] At this moment it is not possible to detect the internalization of 2G-NN16 because it is not a fluorescent molecule. However, when a siRNA fluorescently labeled is bound to the external groups of 2G-NN16, the internalization of fluorescence is observed in PBMCs,[32] primary culture of rat cortical neurones,[33] and human astrocytes,[11] despite the fact that all of these cells are not easily transfectable.

A role in the maintenance of chronic inflammation for CD8+ T cells producing Th17 cytokines has been reported in experimental autoimmune orchitis.[34] In PBMCs from patients with systemic lupus erythematosus, the percentages of both CD4+ IL-23R+ and CD8+ IL-23R+ T lymphocytes were significantly higher than those of control subjects.[35] These data were correlated with significantly higher increases in CD4+ IL-17+ and CD8+ IL-17+ T-cell counts, after ex vivo stimulation in patients with systemic lupus erythematosus. The repression of CD8+ T lymphocytes in Tc17 cells in the presence of 2G-NN16 during the differentiation process suggests a possible application in all of these inflammatory diseases. In vivo studies are necessary to evaluate the potential of this compound for the therapy of inflammatory processes guided by Tc17 lymphocytes.

CONCLUSIONS

The results of this study show that 2G-NN16, a potential drug delivery vehicle, changes the gene expression profiles of CD8+ T lymphocytes. Ribosomal proteins are induced in CD8+ T lymphocytes, thus highlighting the need to characterize the changes induced in the target cell of the molecule transported by the delivery vehicle to avoid opposing effects. 2G-NN16 is also able to inhibit Tc17 cell differentiation, suggesting its potential—to be confirmed—as a drug for the treatment of inflammatory diseases driven by Tc17 cells.

ASSOCIATED CONTENT

Supporting Information

Comparison of 355 genes without exposure to the dendrimer (Supplemental Table 1). This material is available free of charge via the Internet at http://pubs.acs.org

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