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Selective targeting of pro-inflammatory Th1 cells by microRNA-148a-specific antagomirs *in vivo*

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ABSTRACT

In T lymphocytes, expression of miR-148a is induced by T-bet and Twist1, and is specific for pro-inflammatory Th1 cells. In these cells, miR-148a inhibits the expression of the pro-apoptotic protein Bim and promotes their survival. Here we use sequence-specific cholesterol-modified oligonucleotides against miR-148a (antagomir-148a) for the selective elimination of pro-inflammatory Th1 cells *in vivo*. In the murine model of transfer colitis, antagomir-148a treatment reduced the number of pro-inflammatory Th1 cells in the colon of colitic mice by 50% and inhibited miR-148a expression by 71% in the remaining Th1 cells. Expression of Bim protein in colonic Th1 cells was increased. Antagomir-148a-mediated reduction of Th1 cells resulted in a significant amelioration of colitis. The effect of antagomir-148a was selective for chronic inflammation. Antigen-specific memory Th cells that were generated by an acute immune reaction to nitrophenylacetyl-coupled chicken gamma globulin (NP-CGG) were not affected by treatment with antagomir-148a, both during the effector and the memory phase. In addition, antibody titers to NP-CGG were not altered. Thus, antagomir-148a might qualify as an effective drug to selectively deplete pro-inflammatory Th1 cells of chronic inflammation without affecting the protective immunological memory.

1. Introduction

In the murine model of transfer colitis [1], chronic inflammation of the gut can be induced by effector memory T helper type 1 (Th1) cells [2–4]. It is likely that Th lymphocytes are not only able to induce, but also can maintain inflammatory bowel diseases, since treatment of Crohn's disease patients with depleting anti-cluster of differentiation 4 (CD4) antibodies did reduce disease activity significantly [5]. However, therapeutic ablation of $CD4^+$ Th lymphocytes not only in Crohn's disease, but also in other chronic inflammatory diseases, has been abandoned. Anti-CD4 induces long-lasting lymphopenia and immunodeficiency, since also protective Th lymphocytes are depleted [5–7]. Here we describe the *in vivo* use of microRNA-148a (miR-148a)-specific antagomirs (antagomir-148a) for the selective depletion of pro-inflammatory Th1 cells in

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the murine model of transfer colitis.

Recently, we have identified miR-148a as a microRNA (miRNA) specifically upregulated in repeatedly activated Th1 cells, as compared to Th2 and Th17 cells [8]. The T-box transcription factor T-bet and the transcription factor twist-related protein 1 (Twist1), two signature proteins of Th1 cells adapted to chronic inflammation, induce the upregulation of miR-148a. Both Twist1 and miR-148a are highly expressed in effector/memory Th cells isolated from inflamed tissues of patients with chronic inflammatory diseases, including Crohn's disease and rheumatoid arthritis [8,9]. A target of miR-148a is *Bcl2l11*, encoding the pro-apoptotic protein Bcl2-interacting protein (Bim) [8,10–12]. Specific inhibition of miR-148a in repeatedly activated murine Th1 lymphocytes *in vitro*, results in enhanced Bim expression and negatively affects their survival [8].

Antagomirs are 20–25 nucleotides long oligonucleotides complementary to the mature form of distinct miRNAs and conjugated to cholesterol at their 3′ end. They easily penetrate into cells and inhibit the activity of their target miRNAs, both *in vitro* and *in vivo* [13,14]. Here we show that in the murine transfer colitis model, systemic administration of antagomir-148a efficiently targets pro-inflammatory Th1 cells in the inflamed gut, reducing their numbers by 50%, and resulting in a mild but significant amelioration of inflammation. This effect was selective for chronic inflammation, since neither the maintenance nor the functionality of protective $CD4^+$ memory Th cells of the bone marrow (BM) and the spleen were affected in healthy mice that were immunized with NP-CGG to elicit vaccine-like immune responses. Thus, antagomirs represent a novel class of therapeutic molecules that can be used to target pro-inflammatory Th cells, which have adapted to chronic inflammation by the selective expression of distinct miRNAs, here miR-148a.

2. Material and methods

2.1. Mice

C57BL/6 mice were bred and housed under specific pathogen-free (SPF) conditions at the animal facility of the DRFZ Berlin. *Recombination activating gene*-deficient (*Rag1*$^{-/-}$) mice were bred under SPF conditions at Charles River and housed in our animal facility during experiments. All animal experiments were performed in accordance with institutional, state, and federal guidelines. All experiments were approved by the federal state institution “Landesamt für Gesundheit und Soziales” (G0300/11, G0008/13, T0192/10) in Berlin, Germany.

2.2. Cell culture

Naive $CD4^+$ Th cells were isolated from spleens of 6–12 weeks old C57BL/6 mice. First, regulatory T cells were removed by staining with cyanine 5 (Cy5)-coupled anti-CD25 antibodies and subsequent magnetic separation with anti-Cy5 microbeads (Miltenyi Biotec). Afterwards, Th cells were stained with anti-CD4 fluorescein isothiocyanate (FITC) and purified by magnetic-activated cell sorting (MACS®) using anti-FITC microbeads (Miltenyi Biotec). Finally, naive $CD4^+CD62L^+$ T cells were isolated by labeling of Th cells with anti-CD62L microbeads (Miltenyi Biotec). To generate Th1 cells, naive Th cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies (3 μg/ml each, BD Biosciences) in the presence of CD90-depleted, irradiated (30 Gy) splenocytes, as well as anti-interleukin 4 (IL-4) (10 μg/ml, clone 11B11) and the Th1-polarizing cytokine IL-12 (5 ng/ml, R&D Systems). All T cell cultures were conducted in “RPMI complete medium” (pH 7.2), i.e. Roswell Park Memorial Institute-1640 (RPMI) medium (Life Technologies GmbH) containing 10% fetal calf serum (Th. Geyer), 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10 μM β-mercaptoethanol (all from Life Technologies GmbH). To avoid activation-induced cell death, Th cells were removed from anti-CD3/anti-CD28-coated plates before 48 h of stimulation and transferred to new tissue culture plates. After 5 days of culture, viable Th cells were purified by gradient separation with Ficoll histopaque (Sigma-Aldrich), washed with PBS/BSA (i.e. phosphate-buffered saline (PBS) including 0.2% bovine serum albumin (BSA), pH 7.2) and re-stimulated for additional 5 days under the same conditions, except that IL-2 (10 ng/ml, R&D Systems) was added to the cultures. Polarization of Th cells to Th1 lymphocytes was determined by measuring interferon-γ (IFN-γ) expression by flow cytometry following phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation.

2.3. Antagomirs

Lyophilized antagomirs were custom-synthesized according to Krutzfeldt et al. (2005) [13]. Antagomirs were generated by Dharmacon, GE Healthcare, for *in vivo* applications. Purification of both antagomir-148a and antagomir-Scrambled (antagomir-Scr) was performed by high performance liquid chromatography (HPLC) and contained similarly low concentrations of endotoxins, with ≤0.218 EU/mg (endotoxin units per milligram) for antagomir-148 and ≤ 0.2 EU/mg for antagomir-Scr. Antagomir sequences are as follows: antagomir-Scr 5-mU(*)mC(*)mAmCmGmCmAmGmAmU-mUmCmAmUmA-mA(*)mC(*)mG(*)mU(*)-3-Chol [15]; and antagomir-148a 5-mA(*)mC(*)mAmAmAmGmUmUmCmUmGmU-mAmGmUmGmCmAmC(*)mU(*)mG(*)mA(*)-3-Chol [8]. All ribonucleotides were 2-O-methyl modified (mN) and (*) represents a phosphorothioate modification of the backbone. At the 3′-end of the oligonucleotides, a cholesterol (Chol) molecule was added. Lyophilized antagomirs were dissolved in PBS (pH 7.2) at the desired concentration at room temperature for 30 min with slight shaking [14].

2.3.1. Colitis induction and antagomir treatment

Two weeks prior to colitis induction, *Rag1*$^{-/-}$ recipient mice were colonized by oral gavage with fecal bacteria suspensions containing segmented filamentous bacteria (SFB) and *Helicobacter species* (*H. spp.*) [16]. In order to confirm whether the *Rag1*$^{-/-}$ mice were colonized successfully, PCRs of fecal DNA with the following primers were conducted: *H. spp.* Fw: 5′-ctatgacgggtatccggc-3′, Rv: 5′-attccacctacctctccca-3′, SFB Fw: 5′-gacgctgaggcatgagagcat-3′, Rv: 5′-gacggcacggattgttattca-3′. In order to ensure comparable compositions of the intestinal microbiota in antagomir-148a- and in antagomir-Scr-treated (i.e. control) groups throughout the experiments, mice of both groups were co-housed in identical cages during the experiment.

Colitis was induced as published before with small modifications [1]. In brief, repeatedly activated Th1 cells were resuspended in PBS (pH 7.2) in order to transfer 4×10^5 cells into *Rag1*$^{-/-}$ recipients via intravenous (i.v.) injections into the lateral tail vein. On days 2, 4 and 6 after Th1 cell transfer, mice were injected i.v. with 50 mg/kg antagomir-Scr or with antagomir-148a. During ongoing experiments, health conditions of mice were monitored according to the guidelines of the “Landesamt für Gesundheit und Soziales” in Berlin and experiments were terminated according to the same guidelines. The severity of inflammation was evaluated by measuring the length and the weight of the colon to calculate its weight-to-length ratio, which for healthy colons of mice of that age typically is in the range of 35–40 and which increases with higher degrees of inflammation [17–19].

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