

## Differential Expression of Neutrophilic Granule Proteins between Th1 and Th2 Cells

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**Abstract** T helper cell type 1 (Th1) and 2 (Th2) play central roles in immune regulation. To identify the novel genes differentially expressed between Th1 and Th2 cells, CD4<sup>+</sup> T cells were isolated from DO11.10 transgenic mice and induced under Th1 or Th2 conditions. Microarray showed differential expression of neutrophilic granule proteins (NGP) between Th1 and Th2 cells. NGP was first identified as a myeloid-specific granule protein with homology to the cystatin superfamily. Here we confirmed greater expression of NGP in Th2 cells by reverse transcription-polymerase chain reaction and real-time polymerase chain reaction analysis. We also showed that the expression of NGP mRNA had a peak expression after 5 d culture under Th2- but not Th1-biasing conditions. Antibody against NGP was prepared, and in concert with the results of mRNA analysis, the level of NGP protein in Th2 cells detected by Western blot analysis was also higher than that in Th1 cells. GFP-NGP fusion proteins overexpressed in HeLa cells were localized to the cytoplasm. These results suggest NGP is a novel marker distinguishing Th2 from Th1 cells and maybe a novel cytokine secreted by Th2 cells.

**Key words** neutrophilic granule protein; cystatin; Th2 cell

CD4<sup>+</sup> T cells can be divided into T helper cell type 1 (Th1) and T helper cell type 2 (Th2) subsets, which are defined by the secreted cytokines. Th1 cells secrete interferon- $\gamma$  (INF- $\gamma$ ), interleukin (IL)-2, tumor necrosis factor (TNF)- $\alpha$  and TNF- $\beta$ , which are critical for the eradication of intracellular pathogens such as *Listeria monocytogenes* and *Leishmania major*. Th2 cells produce IL-4, IL-13 and IL-5, and they are necessary for inducing the humoral response to combat parasitic helminthes and nematodes. The balance between Th1/Th2 subsets determines the susceptibility to diseases, where the improper development of Th1 cells can lead to autoimmunity, although an overactive Th2 response can lead to allergy and asthma [1,2].

Neutrophilic granule protein (NGP) was first identified as a myeloid-specific granule protein with homology to the cystatin superfamily [3]. Cystatins are natural tight-

binding, reversible inhibitors of cysteine protease [4]. In the immune systems, cystatins can modulate the activity of several components of the immune response. For example, the allergic lung inflammation that characterizes a mouse model of human asthma can be inhibited by the use of an extracellular cysteine protease inhibitor, E64 [5]. Furthermore, parasitic nematodes act on their host's immune system by releasing cysteine protease inhibitors (cystatins) to block effector mechanism [6]. Hartmann *et al.* reported that supernatants from *Acanthocheilonema rviteae* can inhibit CD3-induced proliferation as a result of the filarial cystatin Av17 [7]. Similarly, cystatins from *Onchocerca volvulus*, *Nocardia brasiliensis* and *Trichostrongylus sigmodonitis* inhibit CD3-induced proliferation of white blood cells and splenocytes [8,10]. The effect of cystatins on the immune response is not restricted to T cell proliferation, because it has been shown that parasitic cystatins can change the patterns of Th1 and Th2 response *in vitro*. *In vivo*, chronic filarial parasitic infections have also been associated with a switch to a

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Th2 response in the host, apparently as a result of filarial cystatin activity [11,12]. Filarial cystatins can inhibit the host's response by inhibiting CD3-induced proliferation, and might have additional roles as modulators of the secondary immune response [13].

In the present study, we found the higher expression of NGP gene in Th2 cells than that in Th1 cells by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analysis. In concert with the results of mRNA analyses, the level of NGP protein detected in Th2 cells is also significantly higher than that in Th1 cells. When overexpressed in HeLa cells, GFP-NGP fusion proteins localized to the cytoplasm.

## Materials and Methods

### Animals

DO11.10 and BALB/c mice (6–8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, USA). Mice were kept in a specific pathogen free facility at Chinese Academy of Sciences (Shanghai, China). Mice care and use were in compliance with institutional guidelines.

### Generation of Th1 and Th2 cells

Naive CD4<sup>+</sup> T cells were isolated from DO11.10 spleens using CD4 T cell subset columns (R&D Systems, Minnesota, USA). The resulting cells were determined by CD4 staining. Polarized cell populations were generated by culturing  $0.5 \times 10^6$  cells/ml DO11.10 CD4<sup>+</sup> T cells in a 24-well plate with  $2.5 \times 10^6$  cells/ml irradiated BALB/c antigen-presenting cells (APCs) (2000 rads) in complete RPMI 1640 with the addition of 2 µg/ml OVA peptide and 10 ng/ml IL-2. For differentiation into Th1 cells, 10 ng/ml IL-12 and 10 µg/ml anti-IL-4 were added to the cultures; for Th2 cells, 10 ng/ml IL-4, 10 µg/ml anti-IL-12 and 1 µg/ml anti-IFN-γ were added (all from R&D Systems). The cells were split 1:3 and fed on day 3. For generation of restimulated cells, Th cells were harvested on day 7 after primary activation, washed, and stimulated with OVA peptides (2 µg/ml) at  $5 \times 10^5$  cells/ml with irradiated BALB/c APCs ( $2.5 \times 10^6$  cells/ml).

### Cytokine measurement

For measurement of cytokines produced by the polarized cells, Th1 or Th2 cells were cultured as described above. Supernatants were collected after the restimulation for 48 h and assayed using ELISA kit (BD Bioscience, Bedford, USA).

### RT-PCR and real-time PCR

Total RNA was isolated from Th1 and Th2 cells with Trizol reagent (Invitrogen, Carlsbad, USA) respectively. First-strand cDNA was synthesized using MMLV reverse transcriptase with 4 µg of total RNA. PCR was carried out using an aliquot of first-strand cDNA as a template under standard conditions. For normalization of the amount of RNA loading, RT-PCR of hypoxanthine phosphoribosyltransferase (HPRT) was performed in each RT-PCR reaction as an internal control. The primers used for amplification were as follows: 5'-CTTTGTATTGGTG-GTGGC-3' and 5'-GGTTTCTTGGGTATCCTCT-3' for NGP; 5'-CGAGGTCACAGGAGAA-3' and 5'-TTGGA-AGCCCTACAGA-3' for IL-4; 5'-GCCCTTGACTATAAT-GAG-3' and 5'-GATAAGCGACAATCTACC-3' for HPRT.

Real-time PCR was performed using SYBR green QPCR master mix (Applied Biosystems, Foster City, USA) for IL-4, NGP and HPRT. The reactions were run on the 7900HT fast real-time PCR system (Applied Biosystems). The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by two steps PCR for 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Negative control, in which reverse transcriptase was omitted from the reaction, was run for each sample and reaction for each sample was performed in triplicate. Data were analyzed according to the relative standard curve methods with normalizing the values of HPRT expression in each sample. Melting curves for each PCR reaction were generated to ensure the purification of the amplified products.

### Preparation of rabbit anti-serum and Western blot assay

Each New Zealand white rabbit was immunized subcutaneously at multiple sites on the back with 0.2–0.8 mg of *Escherichia coli*-expressed fusion protein NGP-His in emulsion with complete Freund's adjuvant (1:1, V/V). After 3 weeks, the rabbits were boosted with the protein emulsified in Freund's incomplete adjuvant, followed by another intravenous injection of protein alone in another 3 weeks. Anti-sera were collected 12 d after the last boost and the immunoreactivity titers were monitored by double agar diffusion precipitation performed in 0.8% agarose in phosphate-buffered saline (PBS).

Protein samples (20 µg) were electrophoresized with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis and then transferred onto the polyvinylidene difluoride membrane with BioRad equipments (Hercules, USA). The membrane was blocked with 3% bovine serum albumin for 2 h. After incubating with rabbit anti-sera at 4 °C overnight, the membrane was stained with horse-

radish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, USA) for 1 h. Then the blotting signals were developed using enhanced chemiluminescence detection system (Pierce, Rockford, USA).

### Confocal microscopy analysis of NGP

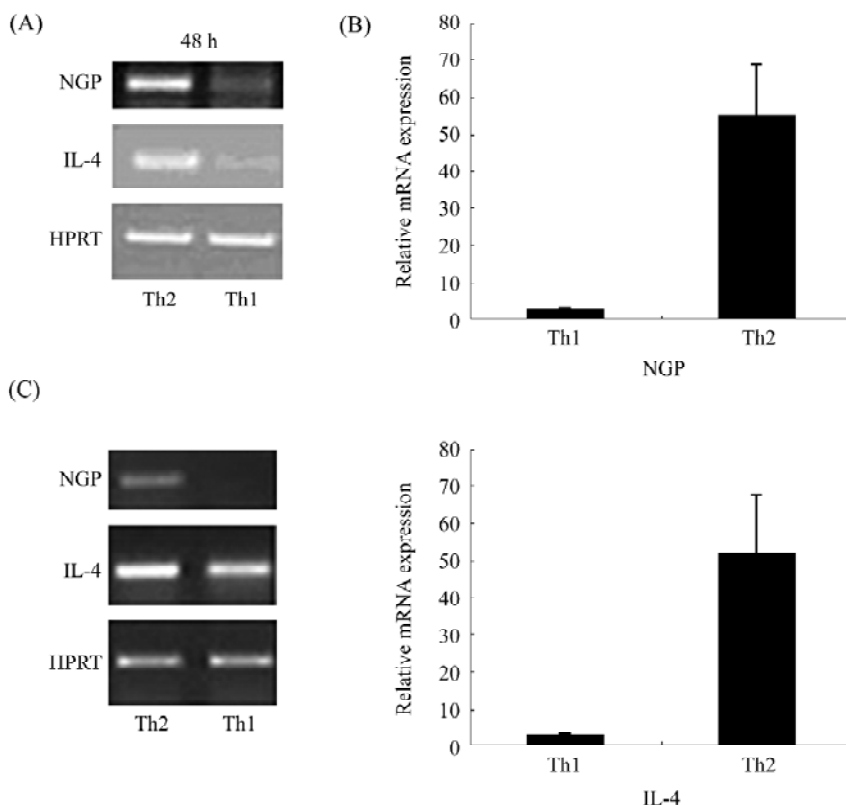
The full length NGP gene was amplified by PCR with forward primer 5'-GGAAGATCTATGGCAGGGCTGTG-GAAG-3' and reverse primer 5'-ACGCGTCGACCG-GAAATTTTCAGGATG-3' using Th2 cDNA as a template. The fragment was fused in the N-terminal of EGFP coding region of the expression vector pEGFP-N1. The recombinant pEGFP-N1-NGP or pEGFP was transfected using Lipofectamine reagent into HeLa cells. The transfected HeLa cells were incubated with 4',6'-diamidino-2-phenylindole dihydrochloride for 1 min. Then the samples were washed briefly in PBS before being observed, mounted onto a microscope slide, and observed by TCS SP2 confocal fluorescence microscopy (Leica, Solms, Germany).

## Results

### NGP gene is differentially expressed in Th1 and Th2 cells

To discover the novel molecules implicated in Th cell differentiation, we generated OVA-specific Th1 and Th2 cells from the DO11.10 transgenic mice *in vitro* and characterized their cytokine secretion by enzyme-linked immunosorbent assay (ELISA). After restimulation with OVA peptide for 48 h, the Th1 cells express a relatively high level of IFN- $\gamma$ , whereas the Th2 cells express a relatively high level of IL-4 (data not shown).

After generation of the OVA-specific Th1 or Th2 cells, we used microarray analysis. The expression of NGP was found to be higher in Th2 than that in Th1 cells (data not shown). We then confirmed the data by RT-PCR and real-time PCR analysis. As shown in **Fig. 1**, there was significantly higher level of NGP mRNA transcription in Th2

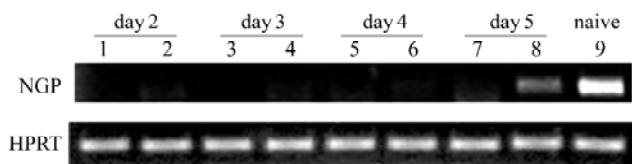


**Fig. 1** Neutrophilic granule protein (NGP) mRNA highly expressed in T helper cell type 2 (Th2) cells

CD4<sup>+</sup> T cells from DO11.10 transgenic mice were stimulated then cultured under T helper cell type 1 (Th1) [interleukin (IL)-12 and anti-IL-4] or Th2 [IL-4 and anti-IL-12, anti-interferon (IFN)- $\gamma$ ] polarizing condition. On day 7, portion of cells were restimulated for 48 h. (A) NGP expression in OVA activated Th cells. (B) Total cDNA were same with (A) and subjected to real-time polymerase chain reaction (PCR) analysis. Results were normalized to hypoxanthine phosphoribosyltransferase (HPRT) abundance. Similar results were obtained in three other independent experiments. (C) NGP expression in anti-CD3 and anti-CD28 activated Th cells.

cells than that in Th1 cells. Because the co-cultures of CD4<sup>+</sup> T cells and APC are inevitably contaminated with granule-containing APC, it could be contributing to NGP expression. Therefore, we purified naive CD4<sup>+</sup> T cells from DO11.10 cells using fluorescence-activated cell sorting and activated them with plate-bound anti-CD3 and anti-CD28 under Th1- or Th2-polarizing conditions. For secondary stimulation, cells were harvested on day 7 after primary stimulation, washed and restimulated at  $1 \times 10^6$  cells/ml with 2  $\mu$ g/ml plate-bound anti-CD3 and 2  $\mu$ g/ml anti-CD28 in Th1- or Th2-inducing culture conditions. We found that NGP expression is induced in Th1 but not the Th2 pathway [Fig. 1(C)].

Furthermore, to examine the kinetics of NGP mRNA expression during primary stimulation of T cells, CD4<sup>+</sup> T cells isolated from DO11.10 transgenic mice were activated in the presence of Th1- or Th2-inducing conditions and RNA was prepared on day 0, 2, 3, 4 and 5. In resting naive T cells, abundant NGP was detected. Furthermore, the expression of NGP gene was undetectable after the antigen stimulation. Interestingly, on day 5, in T cells cultured under Th2- but not Th1-inducing conditions, the expression of NGP gene could be detected again (Fig. 2).

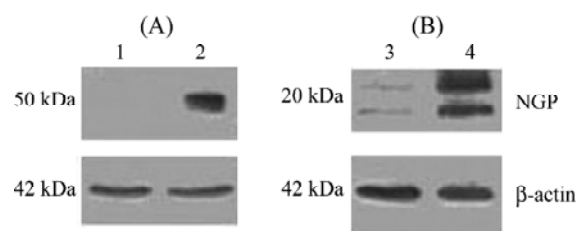


**Fig. 2 Kinetics of induction of neutrophilic granule protein (NGP) transcripts in primary T cells**

CD4<sup>+</sup> T cells isolated from DO11.10 transgenic mice were activated with OVA peptide and irradiated BALB/c antigen-presenting cells (APC) in the presence of T helper cell type 1 (Th1)- or T helper cell type 2 (Th2)-polarizing conditions and reverse transcription-polymerase chain reaction (RT-PCR) analysis carried out on the Th1 cells (1,3,5,7) and Th2 cells (2,4,6,8) harvested on day 2, 3, 4 and 5 after primary activation respectively. Lane 9, naive T cells.

### NGP is expressed more in Th2 cells than that in Th1 cells

To determine whether the NGP is highly expressed in Th2 cells, we expressed the NGP-His fusion proteins in *E. coli* BL21(DE3) and immunized the rabbits three times with the purified NGP-His fusion proteins (data not shown). Then the anti-sera against the NGP-His were confirmed by Western blot analysis. Fig. 3(A) shows that the anti-sera can recognize the cell lysates transfected



**Fig. 3 Western blot analysis of neutrophilic granule protein (NGP)**

Cell lysates were separated in 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto the polyvinylidene difluoride (PVDF) membranes and probed with polyclonal anti-NGP anti-sera. Expression of  $\beta$ -actin was included to ensure equal loading among samples. (A) Lysates from the cells transfected with pEGFP-N1 (lane 1) or pEGFP-N1-NGP (lane 2). (B) Lysates from the polarized T helper cell type 1 (Th1, lane 3) or T helper cell type 2 (Th2 cells, lane 4) restimulated with OVA peptide and irradiated BALB/c antigen-presenting cells (APC) for 48 h.

with plasmid pEGFP-N1-NGP but not the control plasmid pEGFP-N1. In concert with results of the mRNA analyses, Fig. 3(B) shows that the level of endogenous NGP detected in Th2 cells is significantly higher than that in Th1 cells. Two distinct bands might be a result of post-translational modification. This is in line with the result of Moscinski and Hill [3].

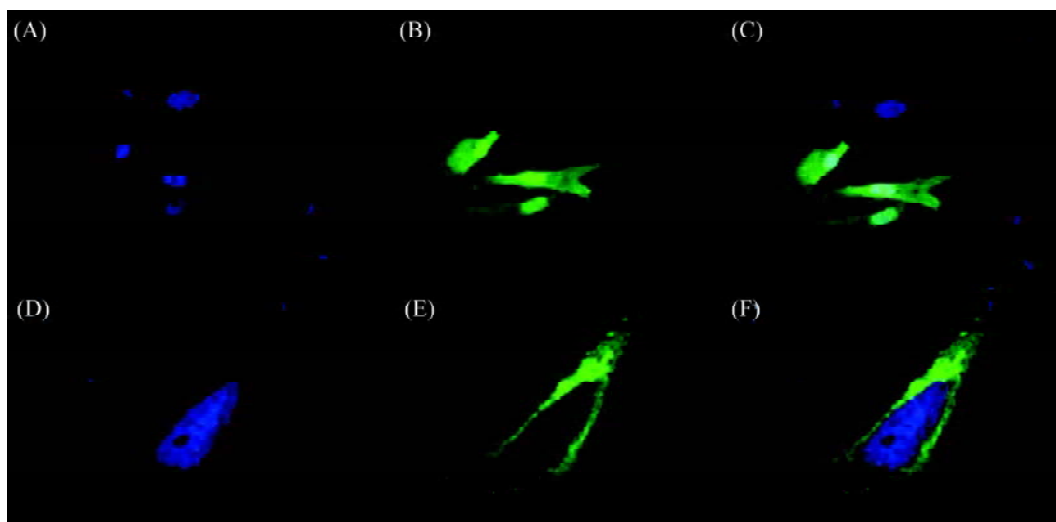
### NGP is localized to the cytoplasm

The localization of NGP was monitored by fluorescence confocal microscopy. In the HeLa cells transfected with pEGFP-N1-NGP, GFP-tagged NGP was localized in the cytoplasm; but when the cells were transfected with pEGFP-N1, the GFP protein was dispersed throughout the cells (Fig. 4). Whether it is localized to the Golgi complex requires further study.

## Discussion

The understanding of helper T cell differentiation has increased in the past several years, but some important questions still remain. We used microarray to analyze the differential expressed genes between Th1 cells and Th2 cells and found that NGP was highly expressed in Th2 cells.

NGP belongs to the cystatin superfamily. It has been reported that nematode cystatins were used by the parasitic nematodes to inhibit proteases involved in antigen processing and presentation, which leads to a reduction of T cell responses [6,8,9,14]. Similarly, we believe that



**Fig. 4** Expression and detection of neutrophilic granule protein-green fluorescent protein (NGP-GFP) fusion protein in transfected HeLa cells

Two-color confocal microscopy analysis of nuclear (blue) and GFP or NGP-GFP (green) was carried out at 72 h after transfection with pGFP (A–C) and pNGP-GFP (D–F). Confocal micrographs showing HeLa cells expressing precursor NGP-GFP fusion protein (D) and GFP protein (B), with the nuclear stained *in vivo* (A and D), and the respective fluorescence signals overlaid (C and F).

the host also exists the similar mechanism to modulate the immune response. In fact, Pierre *et al.* showed that cystatin C found in human, rats and mice were involved in the control of invariant chain degradation and antigen-presentation pathway [15,16]. Recently, the T-kininogen was found to be able to inhibit extracellular regulated kinase-dependent T cell proliferation [13]. Human cystatin salicylic acid (SA) that mainly occurs in saliva and tears [17] can induce INF- $\gamma$  expression in CD4<sup>+</sup> T cells [18]. But whether it exists the similar proteins in immune systems has not been reported.

Here we showed that the level of NGP was higher in Th2 cell than that in Th1 cells by RT-PCR and real-time PCR analysis. We further showed that during the differentiation from naive T cell to effector T cells, the expression of NGP decreased after activation and increased on day 5 in T cells cultured under Th2- but not Th1-inducing conditions. Investigation of the amino terminal sequence of NGP shows two polar amino acids flanking a hydrophobic region, suggesting a signal sequence and the possibility of post-translational modification [3]. The Western blot assay confirmed the level of NGP protein detected in Th2 cells is also significantly higher than in Th1 cells. Two distinct bands found might be a result of post-translational modification. This is in line with sequence analysis. Furthermore, when overexpressed in HeLa cells, GFP-NGP fusion proteins localize to cytoplasm. These

results suggest NGP might be a novel cytokine secreted by Th2 cells. But whether NGP is associated with Th differentiation or function as a novel suppressor to inhibit antigen induced T proliferation need further study.

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