Granule targeting of soluble tumor necrosis factor (TNF) receptor expressed during granulopoietic maturation in murine bone marrow cells

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ABSTRACT. In this experiment, we explored the potential of secretory lysosomes of hematopoietic cells to act as vehicles for immunomodulatory protein delivery at an inflammation site. We investigated whether exogenous soluble TNF-receptor 1 (sTNFR1) could be expressed in primary hematopoietic progenitor cells and become targeted for storage and secretion during granulopoietic differentiation. An sTNFR1 construct with a transmembrane domain (tm) and a cytosol sorting signal (Y) taken from CD63, was retrovirally transduced to lineage-negative murine hematopoietic bone marrow stem/progenitor cells. This process was followed by cytokine-driven granulopoietic maturation. The sTNFR1-tm-Y was found to be synthesized in precursor cells and to persist in mature granulocytes and monocytes/macrophages. Immunofluorescence-localization studies showed a granule pattern of sTNFR1-tm-Y in both precursor and mature granulocytes and secretion to phagosomes after ingestion of bacteria. Immunoelectron microscopy revealed co-localization between the sTNFR1-tm-Y and the primary (azurophil) granule marker myeloperoxidase. Collectively, our results demonstrated granule targeting, storage, and secretion of exogenous sTNFR1-tm-Y constitutively expressed during normal granulopoietic differentiation. These findings support the concept of using storage organelles of circulating hematopoietic cells as vehicles for targeting sites of inflammation with immunoregulatory agents.

Keywords: sTNFR1, secretory lysosomes, granulocytes, murine

Hematopoietic effector cells have a critical role in host defence. Granulocytes are professional phagocytes that release cytolytic granule proteins into phagosomes to kill ingested microorganisms [1]. NK cells and cytotoxic T-lymphocytes (CTLs) eliminate virus-infected and malignant cells through the action of lytic granule proteins such as granzymes and perforin [2, 3]. Each effector cell accumulates bioactive agents in granule containers manufactured during cell differentiation [4, 5]. Granulocyte storage granules are formed sequentially before terminal maturation. First formed are lysosome-like primary (azurophil) granules, which – unlike true lysosomes – lack lysosome-associated membrane protein 1 (LAMP-1) [6, 7]. The proteolytically active primary granules are equipped during biogenesis with fully processed antimicrobial proteins, serine proteases, and lysosomal hydrolases [4, 5]. The proteolytically inactive secondary granules are formed later during differentiation and become equipped with antimicrobial proteins, matrix metalloproteases, and other constituents, mostly as inactive proforms [4]. Characteristically, the lysosome-related organelles in hematopoietic cells co-store cytolytic proteins and lysosomal hydrolases [2, 3]. Furthermore, these organelles are distinguished from conventional lysosomes by a capacity for regulated secretion and therefore are designated secretory lysosomes because of their hybrid nature [3].

We have been exploring the possibility of expressing genes for immunomodulatory proteins and targeting the gene products for storage in secretory lysosomes and inducing co-delivery with granule proteins by regulated secretion. This concept was previously explored in hematopoietic cell lines [8, 9] and is now extended to primary hematopoietic cells.

Granule targeting requires retrieval of cargo proteins in the trans-Golgi network (TGN) or another sorting station to prevent loss by constitutive secretion. The mannose-6-phosphate receptor (MPR) system is used to take lysosome hydrolases and granzymes on the endosomal route from TGN to secretory lysosomes [10, 11]. However, the delivery of many other luminal granule proteins relies largely on unknown targeting mechanisms [5]. In contrast, transmembrane proteins generally depend on defined sorting sequences, which include binding to cytosolic adaptor proteins, recruiting coat, and generating transport vesicles for secretory lysosome delivery [3]. Targeting mechanisms appear not to be specific for endogenous granule proteins,

as we have noted that exogenous non-hematopoietic proteins can also be targeted to secretory lysosomes after gene expression in hematopoietic cell lines [12, 13].

Our ultimate goal in this research is, after *ex vivo* gene transfer to stem/progenitor cells, to take advantage of targeting for storage of anti-inflammatory gene products during myelomonocytic (and other hematopoietic) differentiation. This would be relevant, provided that, the gene product can be expressed in primary cells, is stable during storage, and is released in an active state after migration of the differentiated cells to an inflammation site. It will be important to rule out that the genetic modifications do not inhibit cell migration *in vivo*. Here, we used primary cells instead of cell lines to begin to answer some of these important questions.

Our previous information [8, 14] suggested that storage organelles of hematopoietic cells were potential vehicles for immunomodulators aimed at an inflammation site. Here we investigated the validity of this approach in primary murine hematopoietic bone marrow cultures by retroviral transduction of a soluble-TNF-receptor1 (sTNFR1) gene. The goal was to achieve granule targeting of the gene product during differentiation along the myelomonocytic pathway. Murine hematopoietic progenitor cells were chosen because they can be transduced with higher efficiency than corresponding human cells. Our results demonstrated that sTNFR1-tm-Y (constitutively expressed sTNFR1 with a transmembrane domain (tm) and a cytosol sorting signal (Y)) was targeted to granules in developing granulocytes and monocytes/macrophages. This approach has a therapeutic potential if a beneficial local release of sTNFR1 can be achieved from circulating cells homing to a site of inflammation where TNF needs to be inactivated.

MATERIALS AND METHODS

Materials

Iscove's Modified Dulbecco's Medium (IMDM). RPMI, Dulbecco's MEM (DMEM), and Lglutamine, Gibco BRL (Life Technologies, Grand Island, NY, USA). X-Vivo15 medium, heat-inactivated fetal bovine serum (FBS), BioWhittaker (Verviers, Belgium). Mouse Lineage Cell Depletion Kit and Pre-Separation Filters, Miltenyi Biotec (Bergisch Gladbach, Germany). Retronectin, Takara (Shiga, Japan). Murine SCF, Flt-3 ligand, TPO, IL-3, and GM-CSF, and BSA, Stemcell Technologies (Vancouver, Canada). A polyclonal antiserum against sTNFR1 was obtained by immunization of rabbits and used for immunoprecipitation [15]. Monoclonal anti-sTNFR1 (SC8436) antibody and rabbit anti-mouse CD63 antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A rabbit polyclonal antibody (anti-MPO) was raised against a 14-amino-acid peptide (NTLPKLNLTSWKET) corresponding to the carboxy-terminus of murine myeloperoxidase (MPO). The synthetic peptide was conjugated with Keyhole Limpet Hemocyanin and used to immunize rabbits. The antibodies were affinity-purified against the synthetic peptide. Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 594 chicken anti-goat IgG, DAPI, and ProLong Antifade reagent, Molecular Probes (Eugene, OR, USA).

cDNA constructs

The sTNFR1-tm-Y [8] construct contained cDNA for the extracellular part (sTNFR1), the transmembrane domain from human sTNFR1 with flanking sequences (VKGTEDSGTTVLLPLVIFFGLCLLSLLFIGLMYRYO RWKSKLYSIV) (tm), and the cytosolic tyrosine-sorting lysosome signal (SIRSGYEVM) of CD63 (Y) [2].

Retroviral vectors

The retroviral MSCV vector [16, 17] containing the gene for enhanced green fluorescent protein (GFP) and an internal ribosomal entry site (IRES) was utilized (MIG). The cDNA for human sTNFR1-tm-Y was cloned into the vector, upstream of the IRES. Sequencing results verified that the cDNA was correct. Empty MSCV vector containing only GFP and IRES (mock transduction) or un-transduced cells were used as a control. The retroviral vectors were transfected into the amphotropic Phoenix packaging cell line (from Dr. Nolan, Stanford University School of Medicine, Stanford, CA, USA) with $CaPO_4$ precipitation. Supernatants were harvested after 24-48 hours and passed through a 0.45 μm filter (Millipore, Bedford, MA, USA). Supernatants supplemented with 4 μg/mL protamine sulfate were used for repeated transduction of the ecotropic GP+E86 packaging cell line. High viral titer-stable GP+E86 clones were cultured in 10 cm dishes with 10 mL DMEM-10% FBS at 37°C until 80-90% confluence. The medium was removed and 6 mL of fresh medium added and incubated at 33°C for 24 hours, after which virus-containing medium (VCM) was harvested, filtered, and stored at -80°C [18]. The titers of the harvested supernatants were between 5×10^6 and 6×10^6 TU/mL for the MIG-sTNFR1-tm-Y.

Cell harvest and separation

Bone marrow cells were obtained from femurs and tibias of 8- to 12-week-old, strain B10Q mice (a kind gift from Dr. Holmdahl, Lund University), with approval from the regional animal ethics committee. Lineage-negative bone marrow (L-BM) cells representing hematopoietic progenitor cells were isolated according to the manufacturer's protocol for the mouse Lineage Cell Depletion Kit and resuspended in X-Vivo15 medium. The isolated cells are negative for lineage surface markers, CD5 (T-lineage), CD45R/B220 (B-lineage), CD11b and anti-Ly6G/Gr-1, 7-4 (myeloid lineage), and Ter-119 (erythroid lineage) [19].

Retroviral transduction

Fresh L-BM cells (3×10^5/mL) were prestimulated for 24 hours in serum-free X-Vivo15 medium supplemented with murine SCF, flt3-ligand, and TPO, all at 100 ng/mL. Non-tissue culture-treated, 24-well plates were coated with 10.5 μg/cm^2 of Retronectin for 2 hours, blocked with 2% BSA in PBS for 30 minutes at room temperature, and coated with VCM for 1 hour at 37°C. Prestimulated cells were recovered by centrifugation, suspended in DMEM

medium (50,000/mL) with murine SCF, flt3-ligand, and TPO, all at 100 ng/mL, transferred to the precoated wells, and incubated for 48 hours. Mock transduction with empty MIG vector and untransduced cells was used as control. For granulocyte differentiation, cells were then cultured in IMDM, 20% heat-inactivated FBS, and 0.1 mM 2-mercaptoethanol at an initial concentration of 300,000 cells/mL. When the concentration rose above 1×10^6/mL, the cells were resuspended in fresh medium supplemented with cytokines at 3×10^5/mL. Two different cytokine combinations were used. One combined 50 ng/mL murine SCF and 25 ng/mL human G-CSF (the [SCF, G-CSF] system), which gave rise to a rather pure granulocyte population. The other cytokine combination contained 50 ng/mL murine SCF, 25 ng/mL human G-CSF, 10 ng/mL murine IL-3, and 10 ng/mL murine GM-CSF (the [SCF, GCSF, GM-CSF, IL-3] system). This system resulted in a higher proliferation rate but with substantial macrophage production. Maturation was monitored by morphology from Giemsa-stained cytospin smears.

Biosynthetic radiolabelling

Biosynthetic radiolabelling was carried out as described previously [20].

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were carried out as described previously [8].

Immunofluorescence microscopy

Cells were fixed for 15 minutes on ice and 1 hour at room temperature using 2% paraformaldehyde, permeabilized in cytoskeletal buffer (100 mM KOH, 2 mM $MgCl_2$, 5 mM EGTA, 0.05% (v/v) Triton X-100, and 100 mM PIPES (pH 6.8)) for 12 minutes on ice, and thereafter incubated in blocking solution (PBS containing 1% BSA (w/v), 0.2% Tween 20 (v/v), 0.02% Triton (v/v), and 5% (v/v) goat serum) for 30 minutes at room temperature. Next, cells were incubated with the primary antibodies in blocking solution overnight at 4-8°C. Following washing, cells were incubated with secondary antibodies in blocking solution for 1 hour at room temperature. After washing, the samples were overlaid with ProLong Antifade reagent and mounted. For some samples, after secondary antibody incubation and washing, the samples were stained with DAPI for 1 minute, washed, and mounted. Images were recorded on a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charged-coupled device camera, using a Plan Apochromat 100X objective and a high-numerical-aperture oil condenser.

Immunoelectron microscopy

Cells transfected with sTNFR1 were fixed for 24 hours in 4% paraformaldehyde in 0.1M PHEM buffer (60mM PIPES, 25mM HEPES, 2mM $MgCl_2$, 10 mM EGTA (pH 6.9)) and then processed for ultrathin cryosectioning as previously described [21]. Ultra-thin frozen sections were incubated at room temperature with the indicated antibodies and 10 and/or 15 nm protein A gold as described [21]. After immunolabelling, cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Eindhoven, The Netherlands). For the controls, the primary antibody was replaced by a nonrelevant rabbit antiserum.

Phagocytosis

Phagocytosis of non-opsonized *Streptococcus pyogenes* AP1 mutant BMJ71 [22] was carried out as previously described [23]. Briefly, cells and bacteria at a ratio of 1:10 were pelleted at 37°C, and after 1 minute resuspended in fresh buffer and further incubated at 37°C for 30 minutes to allow phagosome maturation. Phagocytosis was stopped by placing the samples on ice, and the cells were fixed and processed for immunoflorescence microscopy.

RESULTS

Granulopoietic differentiation and transduction efficiency

Exogenous sTNFR1-tm-Y was expressed in murine L-BM cells by retroviral transduction and myelomonocytic differentiation was induced by two different combinations of cytokines, both including G-CSF. The morphology of cultured cells is shown in *figure 1*. In the [SCF, GCSF] system *(figure 1A)*, the culture consisted of 60% myeloid precursor cells and 40% granulocytes on day 7. On day 9, the culture contained 60% granulocytes, 20% macrophage-like cells, and 20% myeloid precursor cells. Later, macrophage-like cells dominated the culture (80–90%). In the [SCF, G-CSF, GM-CSF, IL-3] system *(figure 1B)*, the culture was dominated by myeloid precursor cells and contained approximately 20% mature granulocytes on day 7. On days 9–11, the culture contained approximately 40–50% mature granulocytes, 20–30% macrophage-like cells, and 20–30% myeloid precursor cells, as judged by microscopy counting. Macrophage-like cells dominated this culture on day 14. Thus, the [SCF, G-CSF] system produced a more homogenous granulocyte population than the [SCF, G-CSF, GM-CSF, IL-3] system. However, the latter system showed at least a seven-times higher proliferation rate before day 7 and the cell count was actually decreasing in the [SCF, G-CSF] system after day 7. Differentiation in sTNFR1-tm-Y-transduced, mock-transduced, and un-transduced cells did not differ significantly (not shown).

Flow cytometry analysis of GFP was used to determine the level of transduction *(figure 1)*. In the [SCF, G-CSF] system *(figure 1A)*, both mock-transduced cells and sTNFR1-tm-Y transduced cells gave rise to a GFP signal during granulopoietic differentiation. The GFP signal of day 9 cells was presumed mostly to reflect transduced granulocytes that dominated at this time. Results are also shown for the [SCF, G-CSF, GM-CSF, IL-3] system *(figure 1B)*. Non-transduced cells gave a very low, endogenous green fluorescence signal during differentiation. The transduction efficiency was, in most experiments, approximately 50%, but varied in single experiments from 20% to 90%, as judged by results from cell counting and flow cytometry, regardless of the differentiation system

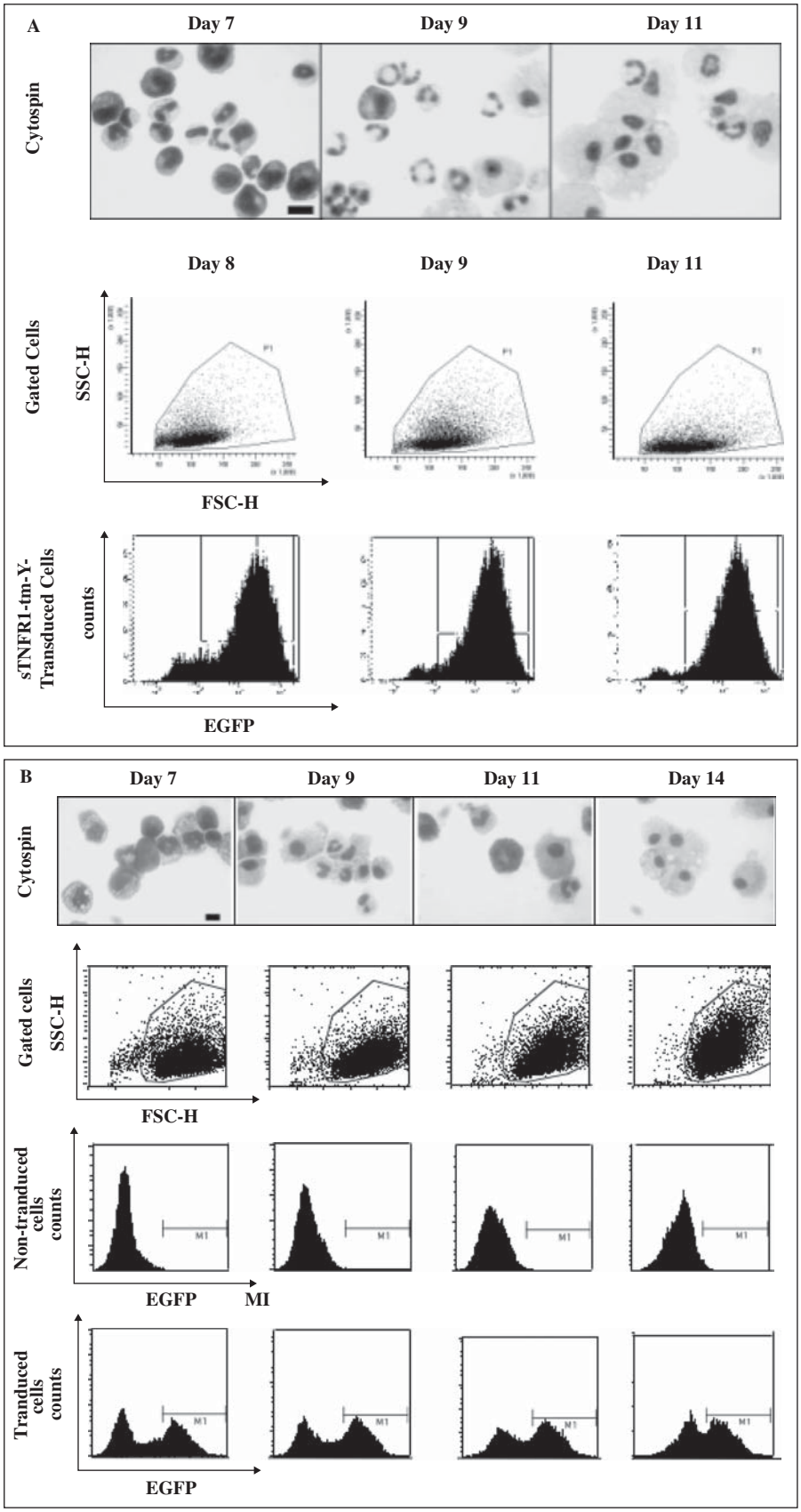

Figure 1

GFP expression during differentiation of transduced cells. Differentiation induced by the [SCF, G-CSF] system (**A**), was dominated by myeloid precursor cells on day 7 and granulocytes on day 9. Differentiation induced by the [SCF, G-CSF, GM-CSF, IL-3] system (**B**) produced a larger cell population with progressive dominance of macrophage-like cells from days 11 to 14. The morphology of cytokine-induced granulopoietic cells by Giemsa staining is shown at different time points. Untransduced cells, mock-transduced cells, and sTNFR1-tm-Y-transduced cells were also analyzed by flow cytometry at various time points of culture. Living immature and mature granulocytes were gated, and histograms of GFP expression are shown. Bar = 10 μm.

used. No significant decrease in fluorescence intensity was seen during culture for 14 days, which was consistent with a continuous biosynthesis of sTNFR1-tm-Y and/or a long half-life for GFP. The overall protein synthesis decreased with granulopoietic differentiation [4]. Collectively, our results demonstrated that the hematopoietic progenitor cells were successfully transduced with the retroviral vector and differentiated into both granulocytes and macrophages.

sTNFR1-tm-Y biosynthesis

The biosynthesis and the steady-state level of sTNFR1-tm-Y were investigated during differentiation in both cytokine systems *(figure 2)*. The steady-state level of sTNFR1-tm-Y was determined by Western blotting *(figures 2A, B)*. Using the [SCF, G-CSF] system, sTNFR1-tm-Y was detected at days 7-10 of culture *(figure 2A)*, when the culture was becoming dominated by granulocytes *(figure 1A)*. A minor component, corresponding to proteolytically released 30-kDa sTNFR1, was detected at all time points in both cytokine systems *(figures 2A, B)*. On day 14, when the cells consisted of macrophages, sTNFR1- tm-Y was still visible *(figure 2B)*. This indicated that monocytes/macrophages also carried the transduced gene. Biosynthetic radiolabelling was used to determine active protein synthesis, leading to detection of newly synthesized 34-kDa sTNFR1-tm-Y *(figures 2C, D)*. Biosynthesis of this protein was observed in both cytokine systems in day 7 cultures *(figures 2C, D)*, which were dominated by precursor cells. Biosynthesis was also observed in day 10 cultures, which were dominated by mature cells. The biosynthesis of sTNFR1-tm-Y was even observed at day 14, when macrophage-like cells dominated *(figure 2C)*. No constitutive secretion of newly synthesized sTNFR1-tm-Y was observed, indicating efficient intracellular retention of this protein *(figures 2C, D)*.

sTNFR1-tm-Y immunofluorescence staining

A sTNFR1 signal was observed in both immature and mature transduced cells. *Figures 3A-3D* show an sTNFR1 signal in a cell with a nuclear shape corresponding to an immature stage of differentiation. Similarly, *figures 3E-3H* show an sTNFR1 signal in a cell with a nucleus corresponding to a mature granulocyte. Both cells were differentiated with the [SCF, G-CSF] system. Macrophages *(figures 3I-3K)* were analyzed at day 14 in the [SCF,

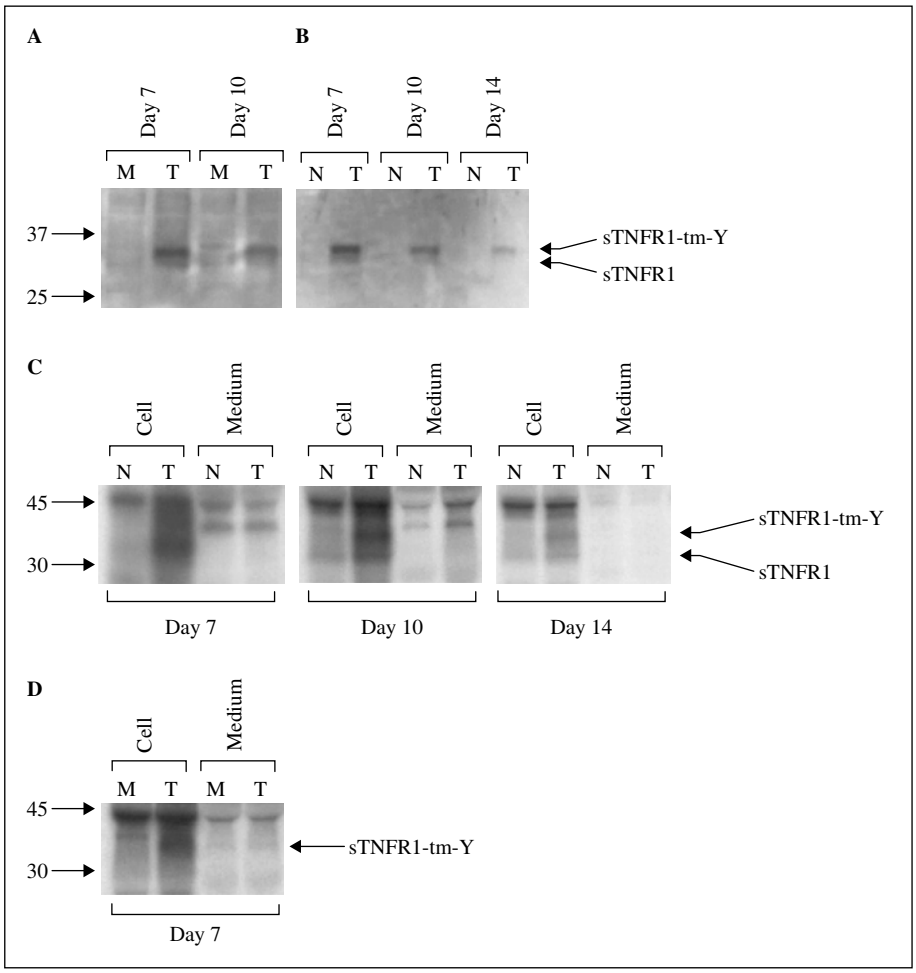

Figure 2

Biosynthetic radiolabelling and Western blotting of sTNFR1-tm-Y. Cell differentiation was induced by the [SCF, G-CSF] system (**A** and **D**) and by the [SCF, GCSF, GM-CSF, IL-3] system (**B** and **C**). For biosynthetic radiolabelling (**C** and **D**) sTNFR1- tm-Y transduced cells were radiolabelled for 5 hours, lysed, immunoprecipitated, and analyzed as described in "Material and Methods". Western blotting (**A** and **B**) was carried on cells at different stages of maturation. The position of sTNFR1-tm-Y and sTNFR1 are indicated with arrows to the right. The position of molecular weight markers are shown to the left. M: mock-tranduced cells, N: untranduced cells T: sTNFR1-tm-Y-transduced cells.

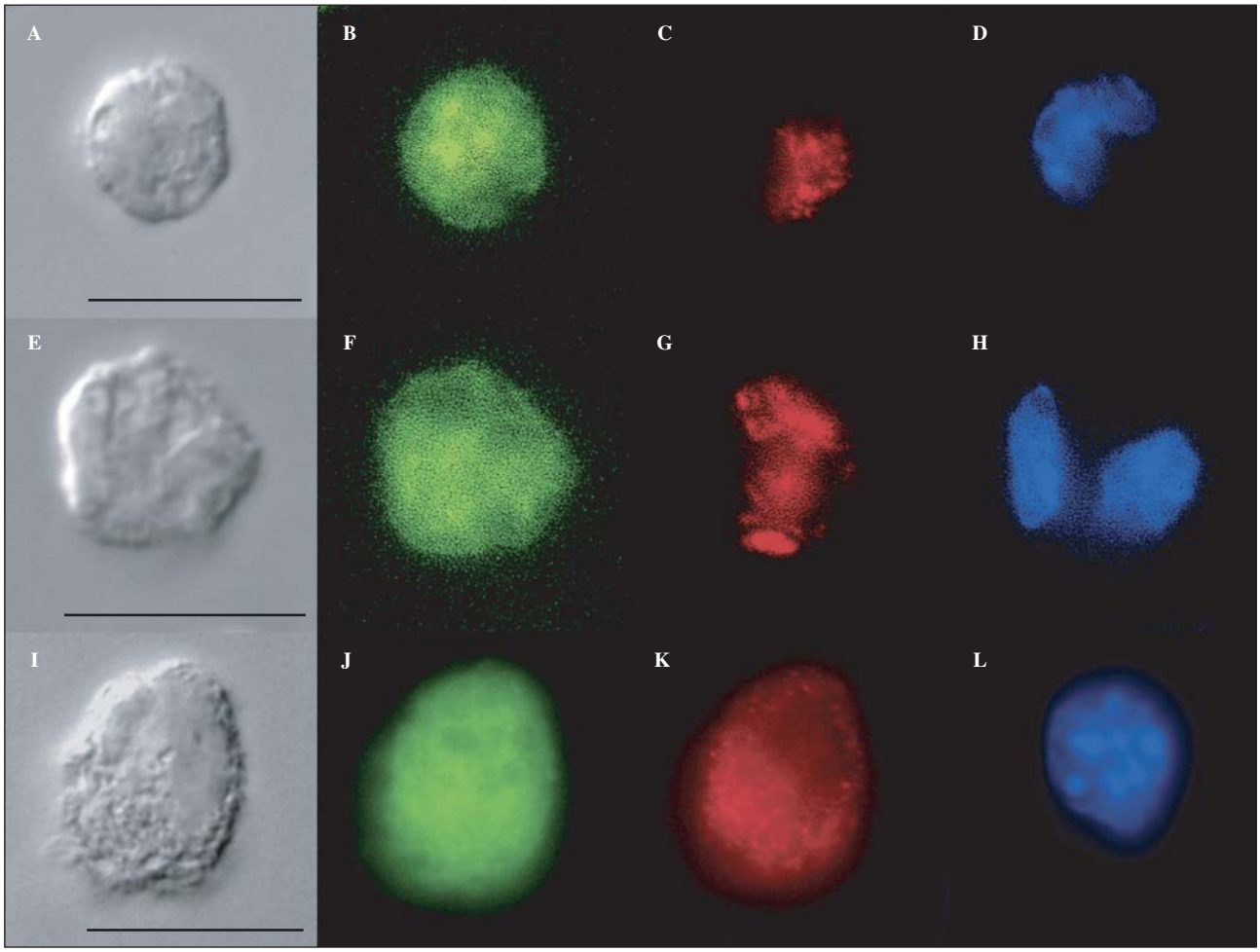

Figure 3

sTNFR1-tm-Y expression in differentiating murine myeloid cells. Lineage-negative murine hematopoietic progenitor cells were transduced using retroviral MIG vector containing the sTNFR1-tm-Y construct. The transduced cells were differentiation-induced by the [SCF, G-CSF] system (**A–H**) and the [SCF, G-CSF, GM-CSF, IL-3] system (**I-L**), and analyzed on day 7 or day 14 of culture with immunofluorescence staining, as indicated. The cells were double stained using a monoclonal mouse anti-sTNFR1 and Alexa Fluor 594 goat anti-mouse secondary antibody followed by nuclear DAPI staining. The GFP signal demonstrates cell transduction of three different cells (B, F, and J). The sTNFR1 signal shows a granular pattern in these cells (**C**, **G**, and **K**). The nuclear shape (D and H) suggests an immature cell in the upper four figures (**A-D**) and a more mature granulocyte in the lower four figures (**E-H**). Mature monocytes/macrophages were analyzed after day 14 in the [SCF, G-CSF, GM-CSF, IL-3] system (**I-L**). The Nomarski images are shown in the leftmost part (**A**, **E**, and **I**). Control experiments with secondary antibody were negative in both untransduced and mock-transduced differentiated cells (not shown). Bar = 10μm.

G-CSF, GMCSF, IL-3] system, and also showed an sTNFR1 signal. Cells with an sTNFR1 signal were also GFP-positive, demonstrating successful transduction *(figures 3B, F, J)*. Approximately 60% (range 50-80% in individual experiments) of the GFP-positive (successfully transduced) cells expressed sTNFR1, as judged by immunofluorescence microscopy. DAPI nuclear staining was used to determine the nuclear shape as an aid to drawing morphological distinction between immature (mononuclear) and mature (polymorphonuclear) cells. No signal was found in non-transduced or mock-transduced cells (not shown).

sTNFR1-tm-Y granule targeting

To demonstrate targeting for granules, we performed immunoelectron microscopy with double immunogold labelling. In these experiments, cell differentiation was induced by the [SCF, G-CSF, GM-CSF, IL-3] system. The sTNFR1-tm-Y was found to co-localize with MPO in granules/vacuoles of precursor cells at day 7, indicating primary granule targeting *(figure 4A)*. At day 10 of culture, a strong co-localization was observed in granulocytes between the sTNFR1 signal and the MPO signal, confirming the targeting of sTNFR1-tm-Y to primary granules *(figure 4B)*. Although, a few untransduced cells showed a positive sTNFR1 signal, this was much weaker than in the transduced cells and did not show a granular pattern (not shown). In conclusion, our data demonstrated targeting of sTNFR1-tm-Y to MPO-positive primary granules during granulopoietic maturation.

Phagosomal redistribution of sTNFR1-tm-Y

Phagocytosis experiments were carried out to determine whether the expressed sTNFR1-tm-Y could be secreted into phagosomes. At day 7 of culture, cells were allowed to phagocytose non-opsonized bacteria *(figure 5)*. Bacteria were efficiently internalized and phagosomes displayed a peripheral, ring-like sTNFR1 signal. This represented

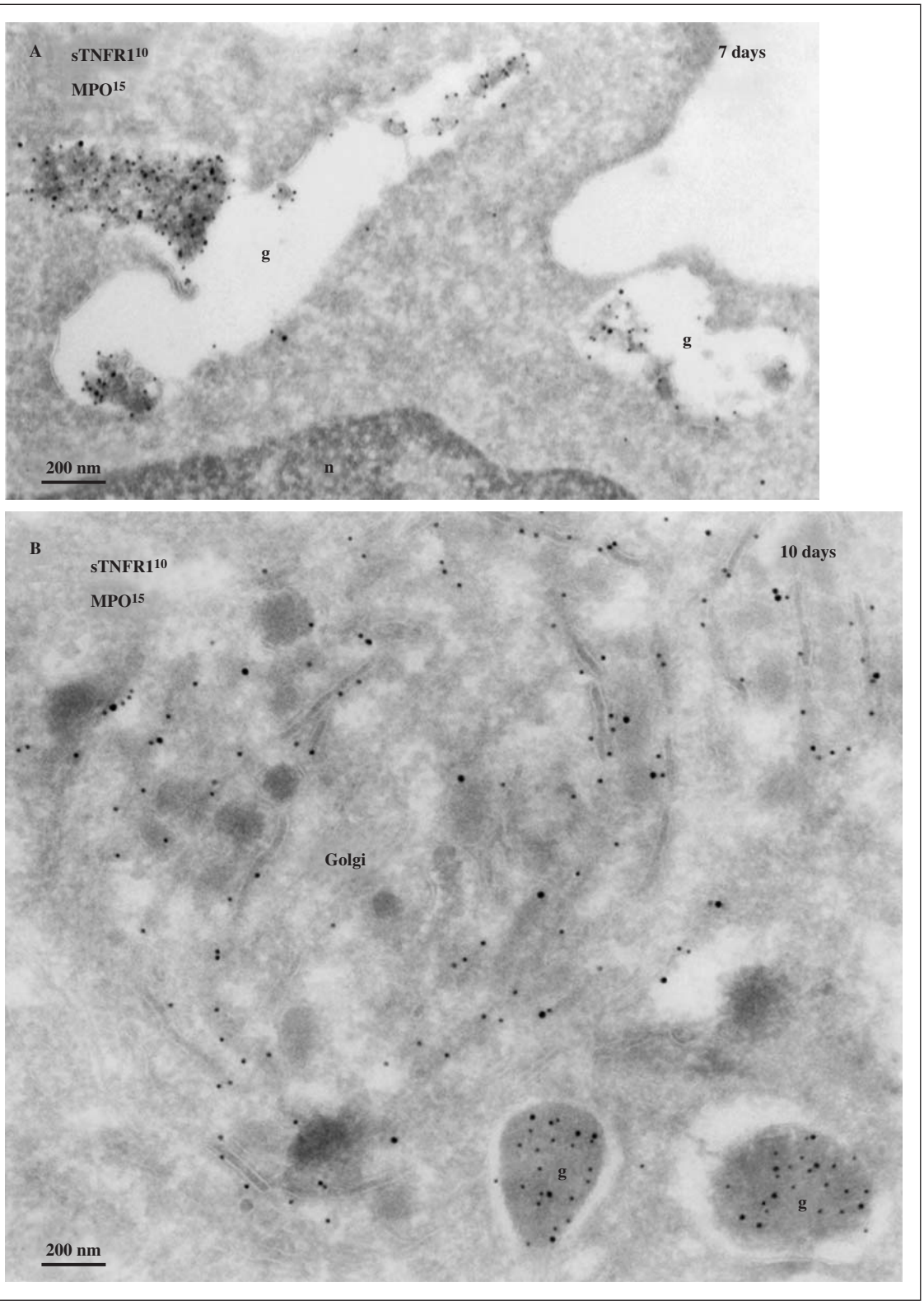

Figure 4

Co-localization of the sTNFR1 signal with MPO (marker for primary/azurophil granules) at different stages of maturation. Mouse granulopoietic cells expressing sTNFR1- tm-Y differentiation induced by the [SCF, G-CSF, GM-CSF, IL-3] system were analyzed on day 7 (A) and day 10 (B) with immunoelectron microscopy. Ultrathin cryosections were double labelled with rabbit anti-TNFR1 (10-nm gold) and rabbit anti-MPO (15-nm gold). In (**A**), two large granules/vacuoles (g) are shown labelled for both sTNFR1 and MPO. (**B**) shows an area of the Golgi and granules (g) both labelled for sTNFR1 and MPO.

phagosome-associated sTNFR1-tm-Y. The data demonstrated that sTNFR1-tm-Y targeted to, and stored in granules could be released into phagosomes.

DISCUSSION

A soluble TNF receptor form was expressed retrovirally in murine hematopoietic cells with the purpose of showing targeting to storage granules during granulopoietic differentiation when subsets of granules are formed. Results from biosynthetic radiolabelling showed that sTNFR1-tm-Y was synthesized in both myeloid precursor cells and in more mature granulocytes. Furthermore, results from Western blotting showed persistence of sTNFR1-tm- Y in mature cells. This suggests that the sTNFR1-tm-Y that was synthesized in myeloid precursor cells and/or mature cells persisted in mature granulocytes. Not only granulocytes but also monocytes/macrophages developed in the cultures, which showed expression of sTNFR1-tm-Y.

We conclude that the sTNFR1-tm-Y synthesized in myeloid precursor cells was able to resist the proteolytic environment of granules to become retained in granulocytes and monocytes/macrophages. Cell proliferation is decreased during terminal granulopoiesis at the transition from promyelocyte to myelocyte, after which secondary and tertiary granules are formed to complete the granulocyte composition [24]. Retroviral expression is dependent on cell division and might diminish in the maturing cells because of quiescence. Persistent biosynthesis of sTNFR1-tm-Y when mature cells dominated the cultures suggested that the retroviral expression was active during terminal differentiation. The constant level of GFP observed during culture was consistent with this notion but could also have been a measure of the long half-life of this protein.

The same sorting mechanisms are probably used for targeting of membrane proteins to both conventional lysosomes and secretory lysosomes. For example, P-selectin has been shown to accumulate in secretory lysosomes using the cytosolic sequences found to be sufficient for conventional lysosome targeting [25]. Primary-granule membrane proteins, such as CD63/LAMP-3, contain a cytosolic tail-sorting signal recognized by the targeting machinery for delivery to granules [26]. The assumption that this cytosolic sorting signal (Y) would also direct targeting of sTNFR1-tm-Y to primary granules was supported. The sTNFR1-tm-Y co-localized with MPO, which confirmed primary granule targeting. The sTNFR1-tm-Y synthesized was assumed to be concentrated at the TGN and recognized by AP3, and, like CD63, bound for primary granules in budding vesicles. The sTNFR1-tm-Y might be translocated to internal vesicles of granules through inward budding, as has been described for CD63 [27]. Eventually, sTNFR1 was proteolytically cleaved and released, at least in part, to the granule matrix, possibly from both the initial limiting membrane location and from the internal vesicles. This was consistent with the presence of both sTNFR1-tm-Y and, to a minor extent, sTNFR1 upon biosynthetic radiolabelling.

The content of a granule reflects which proteins are synthesized during packing and these proteins define the composition of a given granule [28]. Mis-targeting of secondary-granule proteins to primary granules has been observed in leukemia cell lines, when their biosynthesis window has been widened to include the early stages during which primary granule proteins are expressed [13, 29]. The reverse, i.e. widening the biosynthetic window of primary-granule proteins to include the late stages at which secondary granule proteins are synthesized and packed, has not yet been demonstrated. This would be possible with our cell system. However, suitable antibodies for secondary-granule proteins were not available for co-localization between secondary-granule proteins and sTNFR1-tm-Y. Therefore, we could not determine whether the sorting signal corresponding to a tyrosine-based sequence for lysosome targeting facilitated sTNFR1-tm-Y targeting not only to primary granules but also to secondary granules.

The effector proteins stored in granules can be transferred to phagosomes and, in part, to the extracellular environment upon stimulation. Importantly, sTNFR1-tm-Y was redistributed towards phagosomes during phagocytosis. The phagosomal sTNFR1-tm-Y should be derived from granule and endosome compartments, which are alternative membrane sources in phagosome maturation [30, 31]. Our findings indicated sTNFR1-tm-Y to be released from granules by regulated secretion.

Using primary hematopoietic cells for granule targeting is free from the uncertainties inherent in studies with cultured leukemia cell lines, which have a defective ability to express granule protein [32-34] due to differentiation inhibition. However, the *in vitro* system used for granulocyte development is experimental and different from *in vivo* because of non-physiological cytokine concentrations, which may affect the activation state of the cells. Future attempts at *ex vivo* transduction of progenitor cells followed by *in vivo* infusion will be likely to reproduce the differentiation and granule targeting we observed. Several different approaches may thus become feasible for treatment of chronic inflammatory disorders. One is gene delivery by injection at the site of the disease. Another is gene product delivery by circulating cells. Attempts have been reported to suppress renal injury in the Goodpasture syndrome by infusion into sublethally irradiated mice, of marrow cells reconstituted with an IL-1 receptor antagonist (IL-1Ra) [35].

Furthermore, results from other attempts at constitutive systemic expression of IL-1Ra or soluble TNF receptor by secondary circulating cell types in lethally irradiated mice have suggested possible immunoregulatory effects [36-38]. This approach is difficult with truncated proteins such as sTNFR1 because abnormal folding leads to ER retention and protein degradation instead of secretion. In support of this we observed that sTNFR1 was mostly retained in the ER for degradation, and a minor part was constitutively secreted without granule targeting [13]. In the present work, ER retention was diminished by the incorporation of a transmembrane domain and constitutive secretion was prevented by the incorporation of a sorting-signal sequence for secretory lysosome targeting. Therefore, in contrast to other work, we have limited the ER retention, achieved intracellular storage of sTNFR1, and accomplished release by regulated secretion.

An important question is what general advantages there are to using cellular storage organelles rather than other methods of delivery for anti-inflammatory genes without a cell-system intermediate. Delivery of viral or non-viral

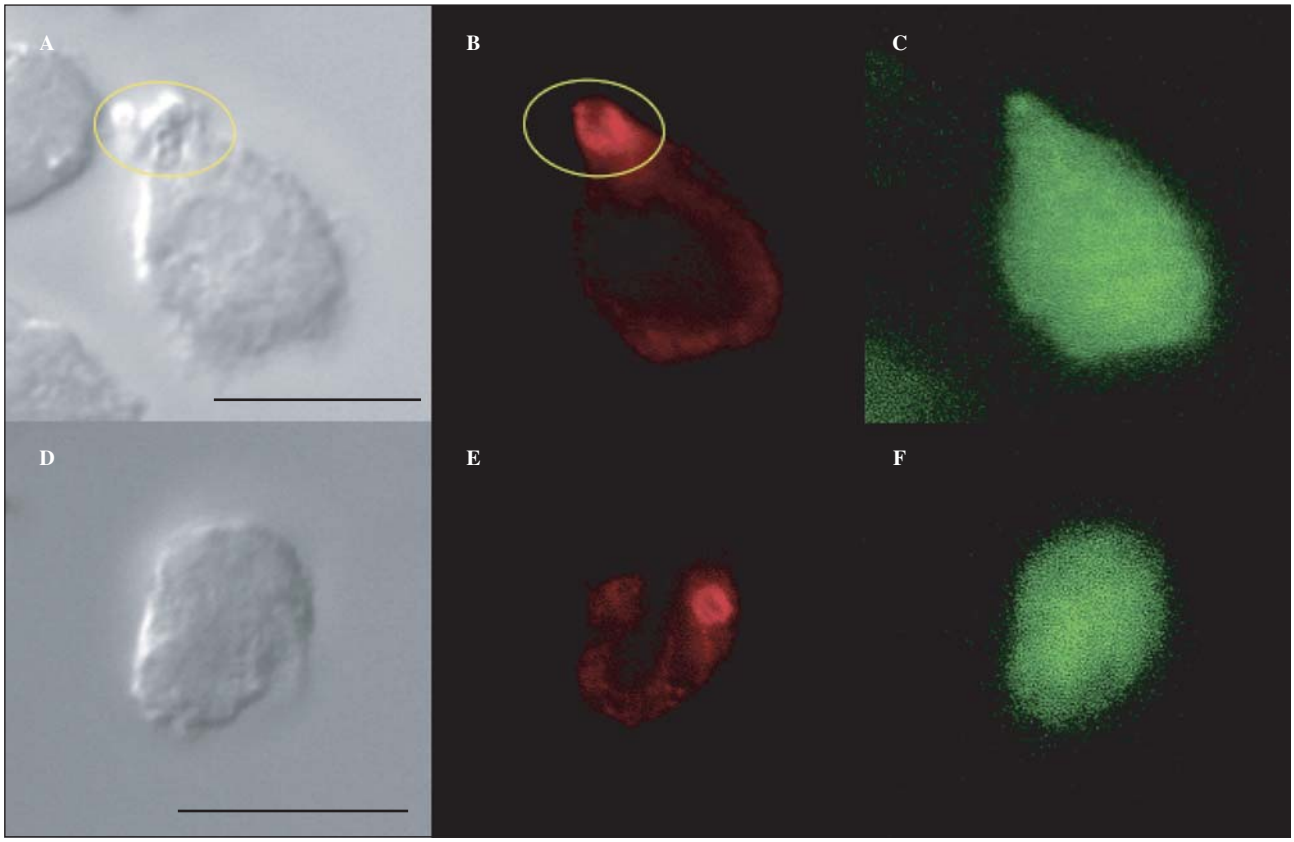

Figure 5

sTNFR1-tm-Y secreted into phagosomes in granulocytes after bacteria ingestion. Cells from day 7 of the cultures expressing sTNFR1-tm-Y and differentiation induced by the [SCF, G-CSF] system were allowed to phagocytose non-opsonized bacteria (10 bacteria/cell) at 37°C. After fixation and permeabilization, cells were stained with a monoclonal mouse anti-sTNFR1, and then stained with Alexa Fluor 594 goat anti-mouse secondary antibody. The first column of photos (**A** and **D**) shows Nomarski images of the cells. Cell-surface-bound and ingested bacteria are seen in A (circle). The second column of photos (**B** and **E**) shows ring-shaped anti-sTNFR1 signals. The ring-shaped structure is localized to the same area as the cell-surface-bound and ingested bacteria, corresponding to a phagosome (**B** and circle). Some cells (**D–F**) also show a ring-shaped phagosome-like sTNFR1 signal without visible bound bacteria. The third column shows the GFP signal of transduced phagocytizing cells (**C** and **F**), indicating a successful transduction. No ring-like structures were seen when incubated without bacteria (not shown). Bar = 10 μm.

vectors directly at the site of inflammation is simple. For example, gene transfer by electrotransfer of naked DNA has been shown to be effective in antigen-induced rheumatoid arthritis [39]. Cell systems still in their infancy are more complex and need *ex vivo* manipulation before their introduction *in vivo*. However, they offer the possibility of gene-product delivery at a beneficial concentration in the entire microenvironment of the inflammatory focus.

Another question is why TNF should be used as the target for modifying myeloid progenitor cells for anti-inflammatory cells. A first reason is that dysregulation of TNF-α is strongly involved in chronic inflammatory disorders [40] and in malignant transformation [41]. However, anti-TNF therapy has side effects because of TNF-α plays an important role in protection against opportunistic infections [42]. As our concept aims at local administration of soluble TNF receptor (or another protein of interest); reduced side effects can be expected compared to systemic administration. Nevertheless, TNF-α may not be the ideal target for attack through myelomonocytic cells such as granulocytes and monocytes/macrophages, as these cells are involved in the first line of defence and require TNF-α for host defence. Results from animal experiments will give answers to such objections. Further, our concept is also applicable to other hematopoietic cells with secretory lysosomes, such as NK-cells and CTLs [2, 3]. These cells have a strong secretory capacity. Finally, exploration of our concept is likely to enhance knowledge of the biology of storage and secretion in hematopoietic effector cells.

Circulating hematopoietic effector cells could be used as carriers in delivery of an immunomodulatory agent to an inflammatory site, provided that this agent is (a) synthesized in a functional form, (b) precisely targeted to an intracellular storage compartment, (c) released by stimulated exocytosis, and (d) delivered in a functional state *in vivo*. Our previous 14 studies showed criteria (a-c) to be fulfilled in leukemia cell lines [8, 9, 14].

In addition, the present results demonstrated that an exogenous protein, sTNFR1-tm-Y, could be targeted to granules when constitutively expressed during granulopoietic differentiation in normal hematopoietic cells, and not only in cell lines. These results further support the potential of using the storage organelles of granulocytes as vehicles for targeting sites of inflammation and malignancy with immunomodulatory agents. Release would be allowed into the phagocytic vacuole and the extracellular space of granulocytes.

Furthermore, this approach, if feasible for CTLs and NK-cells [14], would allow strong agonist-induced regulated secretion to the exterior. Experiments are underway to determine whether sTNFR1 can be delivered in a functional state by circulating hematopoietic cells *in vivo* at the inflammation site.

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