A *trans*-Golgi network golgin is required for the regulated secretion of TNF in activated macrophages *in vivo*

Zi Zhao Lieu*, John G. Lock†, Luke A. Hammond†, Nicole L. La Gruta‡, Jennifer L. Stow†, and Paul A. Gleeson*§

*Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute and ‡Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia; and †Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia

Communicated by Peter C. Doherty, University of Melbourne, Parkville, VIC, Australia, January 6, 2008 (received for review December 8, 2007)

The transmembrane precursor of tumor necrosis factor-α (TNF) exits the *trans*-Golgi network (TGN) in tubular carriers for subsequent trafficking and delivery to the cell surface; however, the molecular machinery responsible for Golgi export is unknown. We previously reported that members of the TGN golgin family are associated with subdomains and tubules of the TGN. Here, we show that the TGN golgin, p230/golgin-245 (p230), is essential for intracellular trafficking and cell surface delivery of TNF in transfected HeLa cells and activated macrophages. Live-cell imaging revealed that TNF transport from the TGN is mediated selectively by tubules and carriers marked by p230. Significantly, LPS activation of macrophages resulted in a dramatic increase of p230-labeled tubules and carriers emerging from the TGN, indicating that macrophages up-regulate the transport pathway for TNF export. Depletion of p230 in LPS-stimulated macrophages reduced cell surface delivery of TNF by >10-fold compared with control cells. To determine whether p230 depletion blocked TNF secretion *in vivo*, we generated retrogenic mice expressing a microRNA-vector to silence p230. Bone-marrow stem cells were transduced with recombinant retrovirus containing microRNA constructs and transplanted into irradiated recipients. LPS-activated peritoneal macrophages from p230 miRNA retrogenic mice were depleted of p230 and had dramatically reduced levels of cell surface TNF. Overall, these studies have identified p230 as a key regulator of TNF secretion and have shown that LPS activation of macrophages results in increased Golgi carriers for export. Also, we have demonstrated a previously undescribed approach to control cytokine secretion by the specific silencing of trafficking machinery.

intracellular trafficking | RNAi transgenic mice | TNF secretion | inflammation | membrane transport

Tumor necrosis factor-α (TNF) is the main proinflammatory cytokine made and secreted by inflammatory macrophages. Early release of TNF in response to LPS or other inflammatory signals enhances the activation and recruitment of T cells and ensures robust innate and acquired immune responses (1). The excessive secretion of TNF is also a prevalent and clinically significant problem in acute inflammation and in chronic inflammatory disease (2). Anti-TNF treatments have shown success in the treatment of rheumatoid arthritis, inflammatory bowel disease, and other conditions (3). Now improved antiTNF strategies that may offer more constrained or cell type specific control of TNF secretion are being sought. This requires identification of molecular mediators of TNF secretion, particularly those that can be targeted to block TNF release.

We have characterized the secretory pathway for TNF in activated macrophages and identified key organelles and components of the trafficking machinery whose expression or function is up-regulated by LPS to support cytokine secretion (4–6). Among these are members of the SNARE family of membrane fusion proteins required at different points along this pathway (5, 6). The transmembrane precursor of TNF is transported from the *trans*-Golgi network (TGN) in tubular carriers that fuse with the recycling endosome as an intermediate compartment, before delivery of TNF to the cell surface for TNF-converting enzyme (TACE)-mediated cleavage and release (5). Although the latter stages of TNF secretion via this pathway are beginning to come to light, an earlier but critical phase of TNF export from the TGN is not yet understood.

One class of components that may regulate membrane transport from the TGN are golgins, long coiled-coil proteins specifically recruited to subdomains of the TGN from which dynamic tubular transport precursors arise (7). There are four human TGN golgins, namely p230/golgin-245, golgin-97, GCC185 and GCC88 (8–13), which all belong to a subfamily of golgins characterized by a C-terminal GRIP domain (14–16). Recruitment of both p230/golgin-245 (p230) and golgin-97 to the TGN is mediated through an interaction with the small G protein Arl1 (17–20); however, these two golgins are localized to distinct membrane domains of the TGN (21). Distinct spatial segregation of p230 and golgin-97 is also reflected in their selective function, each being associated with TGN tubules bearing different cargo molecules. Golgin-97 is associated with distinct membrane extensions of the TGN loaded with E-cadherin (21), and knockdown of golgin-97 selectively blocks exit of E-cadherin cargo from the TGN in transfected HeLa cells (21). However, p230 labels tubular extensions devoid of E-cadherin and loaded instead with recombinant TNF when expressed in transfected HeLa cells. The role of these golgins in TNF exit in HeLa cells and their propensity for selective transport of cargo could have profound implications in activated macrophages where LPS induces the rapid synthesis of proinflammatory cytokines. Here, we demonstrate a physiologically regulated function of p230 *in vivo* and its essential role in cytokine secretion in mouse macrophages.

Results

TGN Golgin, p230, Is Required for TNF Secretion in HeLa Cells. Golgins mark different subdomains of the TGN, and tubules arising from these subdomains have different golgins associated with them (7, 21, 22). We have shown that p230 is associated with tubular extensions of the TGN loaded with TNF in HeLa cells (21). To determine whether p230 was required for postGolgi export of TNF, HeLa cells were depleted of p230 by using siRNA and then transfected with YFP-TNF. p230 was depleted to >75% in siRNA-transfected cells, whereas the related TGN golgin, golgin-97, was unaffected (Fig. 1*d*). In both control and p230-depleted cells, YFP-TNF showed intracellular perinuclear

Author contributions: Z.Z.L., L.A.H., J.L.S., and P.A.G. designed research; Z.Z.L., J.G.L., and L.A.H. performed research; N.L.L.G. contributed new reagents/analytic tools; J.L.S. and P.A.G. analyzed data; and J.L.S. and P.A.G. wrote the paper.

The authors declare no conflict of interest.

§To whom correspondence should be addressed. E-mail: pgleeson@unimelb.edu.au.

This article contains supporting information online at www.pnas.org/cgi/content/full/0800137105/DC1.

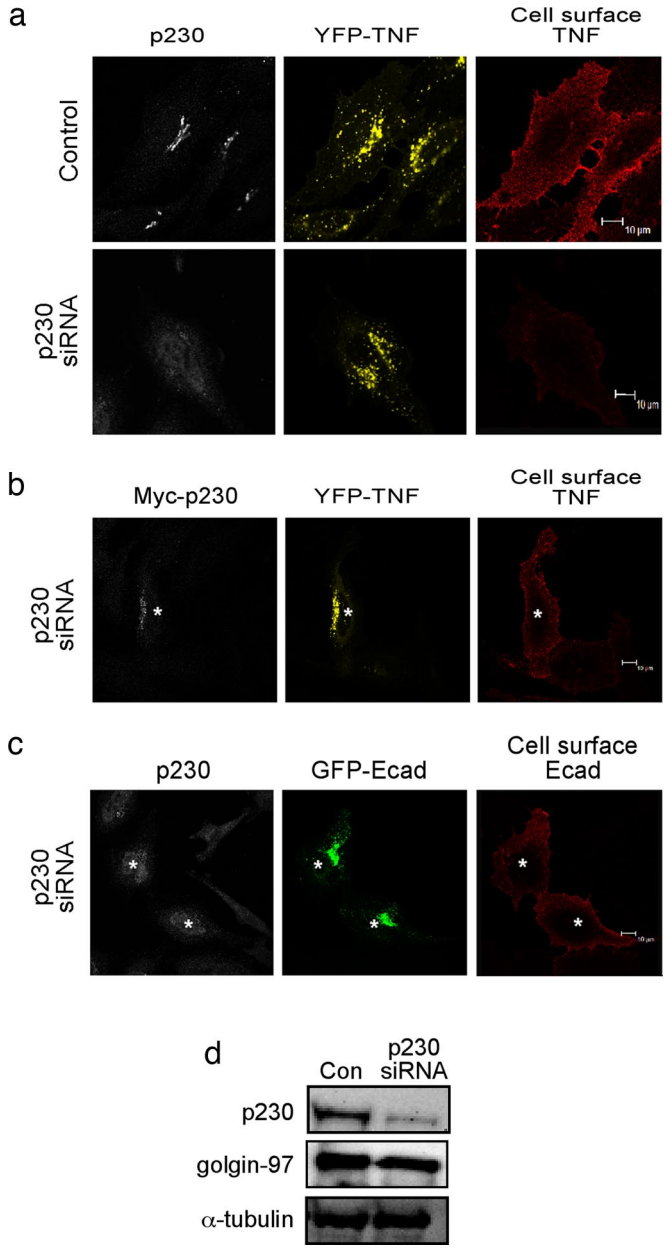

Fig. 1. TNF trafficking is inhibited by silencing p230 in HeLa cells. (*a–c*) HeLa cells transfected with control siRNA or p230 siRNA for 48 h and then transfected a second time with YFP-TNF (*a* and *b*) and GFP-Ecad (*c*) for a further 24 h. In *b*, myc-tagged full length p230 (myc-p230) was cotransfected with YFP-TNF. Monolayers were then incubated with TACE inhibitor for 2 h, fixed in paraformaldehyde and cell surface TNF stained with rabbit anti-TNF antibodies followed by Alexa647-conjugated anti-rabbit IgG. Monolayers were then permeabilized and stained with human anti-p230 antibodies followed by Alexa594-conjugated goat anti-human IgG (*a* and *c*) or monoclonal anti-myc antibodies followed by Alexa568-conjugated anti-mouse IgG (*b*). (*d*) HeLa cells were either mock transfected (Con) or transfected with p230 siRNA for 72 h, as indicated, and lysed in reducing buffer and extracts subjected to SDS/PAGE on a 4–12% gradient polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with affinity-purified rabbit anti-p230 antibodies by using chemiluminescence detection. The membrane were then stripped and reprobed with anti-α-tubulin, followed by anti-golgin −97 antibodies. (Scale bars, 10 μm.)

Golgi-like staining (Fig. 1*a*), as reported (5, 21, 23). To detect TNF at the cell surface, a TACE inhibitor was included to block proteolytic release of surface TNF. Cell surface TNF was readily detected on nonpermeabilized control cells incubated with

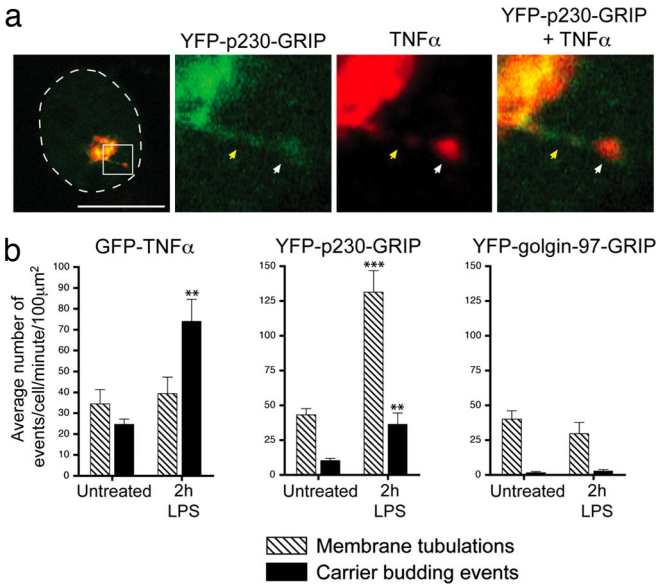

Fig. 2. TNF in p230 labeled tubules leaving the TGN in live macrophages. (*a*) RAW macrophages were transfected with YFP-p230-GRIP and then fixed in 4% paraformaldehyde. Fixed macrophages were permeabilized and stained with rabbit anti-TNF antibodies. Images show YFP-p230-GRIP (green) labeled tubule (yellow arrow) containing a bolus (white arrow) of TNF (red) extending from a region of the TGN. (*b*) Quantification of events observed in live RAW macrophages transfected with either GFP-TNF, YFP-p230-GRIP or YFP-golgin-97-GRIP. In LPS stimulated macrophages, a 3-fold increase in GFP-TNF carrier budding from the Golgi is observed. LPS stimulation also results in a nearly 3-fold increase in YFP-p230-GRIP tubulations and carrier budding, while having no effect on the activity of YFP-golgin-97-GRIP. The number of cells analyzed for each condition ranged from 20 to 40. Data are expressed as mean ± SEM. ***, $P < 0.0001$; **, $P < 0.01$ by unpaired two-tailed *t* test. (Scale bar, 10 μm.)

TACE inhibitor (Fig. 1*a*); however, there was very little or no surface TNF detected on the majority (>80%) of p230 siRNA-transfected cells. In contrast, and as expected from our previous findings, the membrane cargo E-cadherin protein was efficiently transported to the plasma membrane of p230-depleted cells (Fig. 1*c*). Depletion of another TGN golgin, GCC88, has no effect on cell surface transport of TNF [supporting information (SI) Fig. 5], demonstrating that the block in TNF export was p230-specific.

To rule out the possibility of siRNA off-target effects, p230-depleted HeLa cells were transfected with a myc-tagged full-length p230 construct (myc-p230) to determine whether overproduction of wild-type p230 would rescue the observed block in TNF transport. Staining of TNF was observed on the surface of all such transfected cells (>20 cells; Fig. 1*b*), indicating that myc-p230 rescued the block in TNF transport out of the TGN.

LPS-Regulated Membrane Tubulation and TNF Transport in Live Macrophages. Having established a role for p230 in the post-Golgi export in HeLa cells, we next examined the relevance of these findings in macrophages. LPS-stimulated RAW264.7 macrophages were examined for the relationship between p230 and TNF at the TGN. Macrophages were transiently transfected with GFP-TNF and/or with YFP-labeled GRIP domains of p230 or golgin-97. Live imaging detects tubules that emerge from the TGN, structures not readily detected in fixed cells (21). TGN-derived tubules and budding carriers were viewed in live macrophages (SI Movies 1–4) and also analyzed by immunolabeling in a series of fixed cells. Typically, endogenous TNF or GFP-TNF was seen emerging from the TGN as a bolus in tubules labeled with the YFP-p230$_{GRIP}$ (Fig. 2*a*). The transport of TNF from the TGN to the recycling endosome

involves the SNARE complex of syntaxin6/syntaxin7/Vti1b (6), and accordingly p230 tubules can be seen colabeled with syntaxin6 (SI Fig. 6). Neither endogenous TNF nor GFP-TNF in macrophages were associated with golgin-97-labeled tubules (data not shown), thus TNF transport is selectively accomplished by p230-labeled tubules and carriers. This selectivity emulates the same combinations of cargo and golgins on tubules recorded in transfected HeLa cells where TNF was also seen exclusively in p230-labeled tubules (21). Thus, it appears that TNF trafficking and secretion in activated macrophages relies preferentially on 230-labeled tubules.

p230 Tubule Formation Is LPS-Regulated. Upon activation with LPS, macrophages undergo a dramatic increase in exocytic trafficking activity and up-regulate the expression of key components of their trafficking machinery to accomplish this (6). There is a significant increase in the number of GFP-TNF-labeled tubules and carriers emerging from the TGN in LPS-activated cells, reflecting the heightened secretory capacity of these cells (Fig. 2*b*). The activities of $p230_{GRIP}$- and $golgin\text{-}97_{GRIP}$-labeled membranes on the TGN were monitored in live cells before and after treatment with LPS. We counted the relative frequency of p230- or golgin-97-labeled tubules emerging from the TGN in live activated or resting macrophages. In the absence of LPS, the TGN gives rise to approximately equal numbers of p230 or golgin-97-labeled tubules and carriers (Fig. 2*b*). After LPS activation, the number of golgin-97 tubules/carriers did not change, but there was a marked (≈3-fold) increase in the number of p230 tubules emerging from the TGN and an equivalent increase in p230-labeled budding events (Fig. 2*b*). Thus, there is a selective increase in p230 tubules and carriers accompanying cell activation and the need to secrete TNF.

To test whether p230 has a functional role in TNF secretion in macrophages, we used a vector-based micro RNA (miRNA) system to deplete intracellular p230. RAW macrophages were transfected with the BLOCK-iT Pol II miR RNAi expression vectors, which contain a GFP reporter gene, and the extent of p230 depletion was determined 96 h after transfection by immunofluorescence. Very little p230 was detected in GFP+ macrophages transfected with miRNA target sequence one (miRNA-1) (Fig. 3*a*) or two (miRNA-2) (data not shown), whereas control miRNA had no apparent effect on endogenous p230 levels (Fig. 3*a*). On LPS activation, both control and p230-depleted macrophages showed strong perinuclear staining for TNF (Fig. 3*a*), demonstrating the production of precursor TNF in LPS-stimulated macrophages. However, whereas control miRNA macrophages showed high levels of surface TNF by immunofluorescence and flow cytometry, p230 miRNA-transfected macrophages showed very little surface TNF staining (Fig. 3 *b* and c). The level of surface TNF on p230-depleted macrophages was <10% of control macrophages (Fig. 3*c*). A dramatic reduction of surface TNF was also observed with a second independent miRNA p230 target (data not shown) and an siRNA p230 target (SI Fig. 7), thereby ruling out off-target affects of the p230 RNAi. The block in TNF trafficking was not observed when a different TGN golgin, namely GCC185, was depleted with miRNA (Fig. 3*b*). To determine whether all cell surface components were affected by p230 depletion in RAW cells, we also analyzed surface MHC class II expression, which increases after LPS stimulation of macrophages. Surface MHC class II expression was elevated to similar levels in both control and p230-depleted LPS-stimulated macrophages after LPS treatment (data not shown). Therefore, as for HeLa cells, p230 depletion results in a block of specific cargo from the Golgi apparatus of macrophages. Collectively, these findings demonstrate that p230 is an essential component of the tubules at the TGN required for post-Golgi transport of TNF.

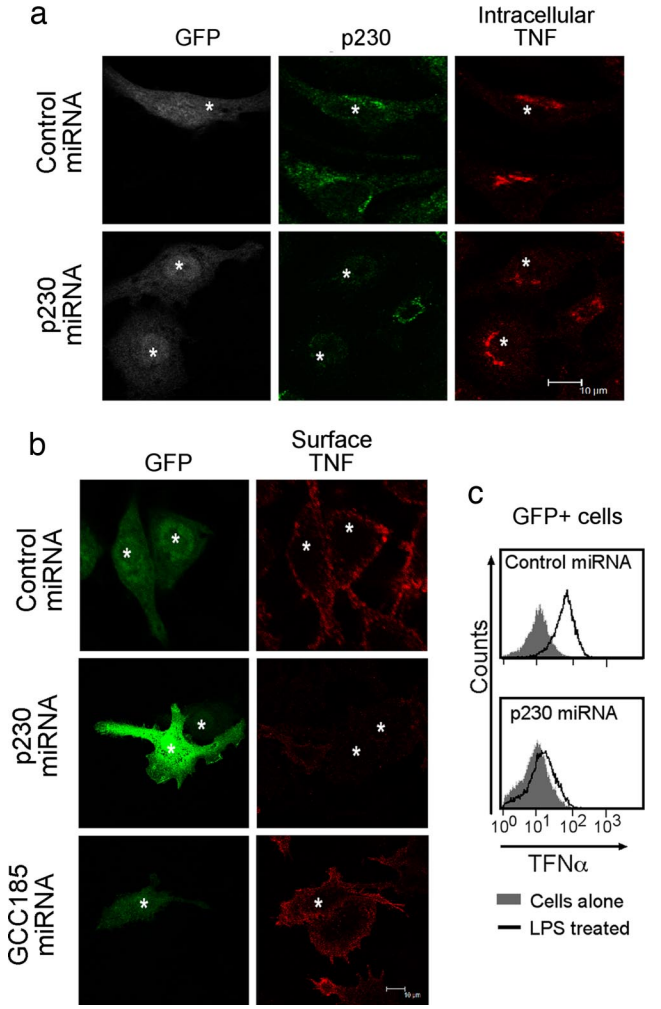

Fig. 3. Post-Golgi TNF trafficking is inhibited by silencing p230 in stimulated RAW macrophages. RAW macrophages were transfected with control miRNA or p230 miRNA-1, as indicated, for 96 h and treated with 100 ng/ml of LPS in serum-free RPMI medium 1640 at 37°C for 2 h in the presence of the TACE inhibitor. Stimulated macrophages were then fixed in 4% paraformaldehyde. (*a*) Fixed macrophages were permeabilised and stained with human anti-p230 antibodies followed by Alexa647-conjugate goat anti-human IgG and with rabbit anti-TNF antibodies followed by Alexa568-conjugated anti-rabbit IgG. (*b* and *c*) Fixed macrophages were analyzed for cell surface TNF by staining with rabbit anti-TNF antibodies followed by Alexa568-conjugated anti-rabbit IgG. Representative flow cytometry plots of GFP+ cells shown in *c*. (Scale bars, 10 μm.)

Peritoneal Macrophages from Transgenic Mice Expressing p230 miRNA Are Blocked in TNF Secretion. To determine whether depletion of p230 could block TNF secretion *in vivo*, we generated transgenic mice expressing RNAi. We silenced p230 in mice by retroviral transduction and bone marrow transplantation. p230 and control miRNA constructs were cloned into a murine stem cell vector-based retroviral vector, retrovirus produced and bone marrow cells transduced with the recombinant retrovirus in the presence of IL-3, IL-6, and SCF. Two days after infection with retrovirus, transduced stem cells were injected into sublethally irradiated recipient mice, and peritoneal macrophages were analyzed 8–10 weeks after the transplant. GFP+ macrophages from control and p230 miRNA-expressing mice showed the characteristics of wild-type macrophages including extensive membrane ruffling after LPS stimulation (data not shown). p230 miRNA resulted in depletion of p230 in peritoneal macrophages of transgenic mice (Fig. 4*a*), whereas strong p230 staining was present in control miRNA peritoneal macrophages (Fig. 4*a*). The staining patterns of other TGN golgins,

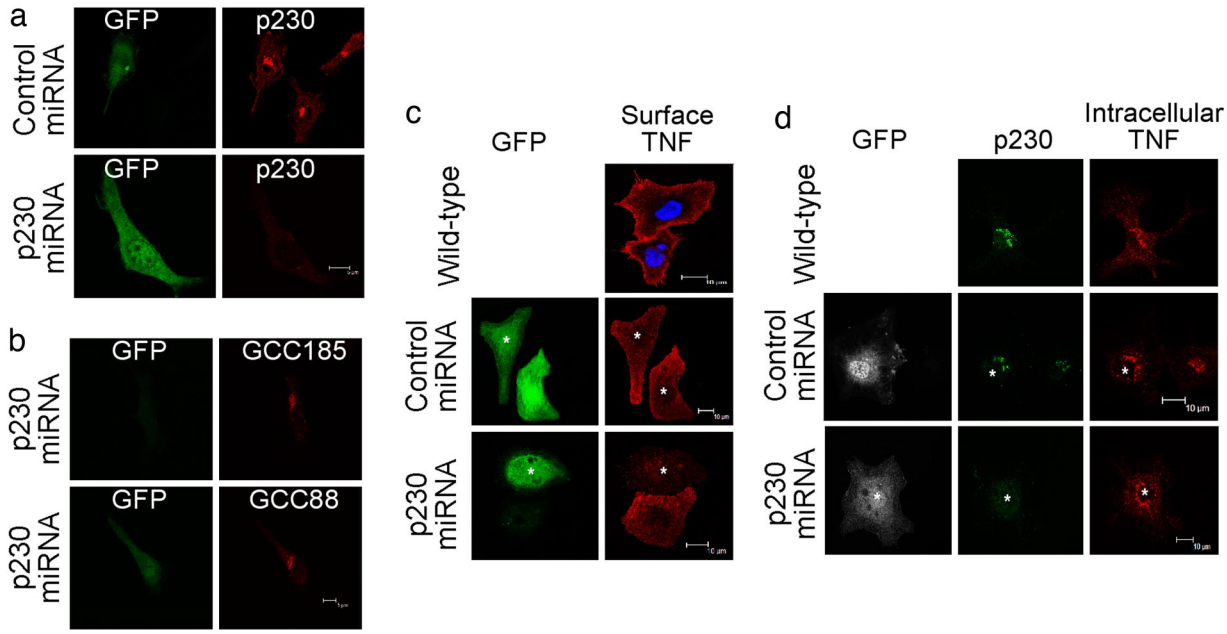

Fig. 4. Peritoneal macrophages from transgenic mice expressing p230 miRNA are depleted in p230 and impaired in TNF secretion. Peritoneal macrophages obtained from either empty miRNA vector (control) or p230 miRNA retrogenic mice were fixed in 4% paraformaldehyde and stained with anti-human p230 antibodies, followed by goat Alexa594 conjugated anti-human IgG (*a*) or rabbit anti-human GCC88 antibodies or rabbit anti-human GCC185 antibodies, followed by goat Alexa568 conjugated anti-rabbit IgG (*b*). (*c* and *d*) Peritoneal macrophages from control and p230 miRNA transgenic mice were activated with 100 ng/ml of LPS in the presence of the TACE inhibitor for 2 h. Macrophages were fixed in 4% paraformaldehyde and cell surface TNF detected in nonpermeabilized cells by using rabbit anti-mouse TNF antibodies, followed by goat Alexa568 conjugated anti-rabbit IgG. (*d*) For intracellular TNF detection, macrophages were fixed and permeabilized, and stained with rabbit anti-mouse TNF antibodies, followed by goat anti-rabbit Alexa 568 conjugated antibodies. (Scale bars, 10 μm.)

namely GCC88 and GCC185, were unaffected by the depletion of p230 (Fig. 4*b*). After LPS stimulation in the presence of TACE inhibitor, intracellular TNF was readily detected in both control and p230 miRNA macrophages, whereas there was a marked difference in the level of surface TNF (Fig. 4 *c* and *d*). Control miRNA GFP+ macrophages had high levels of surface TNF, whereas there was only a low level of surface TNF in the p230 miRNA macrophages. Surface TNF was quantified by confocal microscopy, as described in *Materials and Methods*, and GFP^{bright} p230 miRNA macrophages (n = 25) showed no increase in surface fluorescence after LPS treatment compared with resting macrophages, whereas GFP^{dull} macrophages showed an increase in surface fluorescence to ≈25% the level measured in control miRNA macrophages. These findings indicate that the level of expression of the miRNA construct is sufficient to inhibit p230 *in vivo*, and moreover, p230 depletion results in a block in post-Golgi transport of TNF. Furthermore, these analyses demonstrate the applicability of miRNAs to deplete cellular components and disrupt membrane trafficking pathways *in vivo*.

Discussion

There are multiple transport pathways from the TGN to the cell surface, and the particular transport route used depends on the individual cargo (24). Our study has identified the TGN golgin, p230, as a key regulator of TNF secretion by activated macrophages *in vivo* and, furthermore, has demonstrated an approach to control secretion by the specific silencing of trafficking machinery. The discovery that depletion of p230 selectively blocks post-Golgi TNF transport and secretion in either transfected HeLa cells or LPS-stimulated macrophages indicates that TNF uses a p230-dependent transport pathway for export in a variety of different cell types. TNF production by immune cells is induced by inflammatory stimuli and the dramatic up-regulation of p230-tubules upon LPS stimulation of macrophages highlights the importance of these structures for TNF secretion. p230 is present in macrophages as an abundant soluble and peripheral membrane protein. Because the expression of p230 is not up-regulated by LPS (data not shown), the increase in the number of p230 tubules after LPS stimulation is likely to be a consequence of enhanced recruitment of the existing pool of p230 from the cytosol to the TGN. Silencing of p230 expression resulted in a block in anterograde transport of TNF. This block in TNF transport was a specific consequence of the absence of p230, because (*i*) the silencing of p230 had no effect on the levels of the Arl1-dependent TGN golgin, golgin-97; (*ii*) staining for *cis*- and *trans*-Golgi markers showed no obvious perturbation in Golgi organization; (*iii*) anterograde transport of E-cadherin in p230-depleted cells was normal indicating a functional Golgi apparatus; (*iv*) independent miRNA and siRNA targets produced the same transport defect of TNF; (*v*) RNAi silencing of either GCC88 in HeLa cells or GCC185 in primary macrophages did not result in a block in transport of TNF; and (*vi*) overexpression of exogenous full-length p230 rescued the transport defect in siRNA treated HeLa cells. Together with the physical association of p230 with TNF-loaded tubules emerging from the TGN, the results demonstrate that p230 is directly involved in the selective anterograde transport of TNF out of the TGN.

TGN golgins have been shown to play a role in defining the characteristics of TGN membranes (7). The two TGN golgins, p230 and golgin-97, depend on Arl1 for membrane recruitment (17–19, 25). Our previous analyses showed that different cargoes are selectively packaged into different Golgi tubules, and that these tubules bud off to form transport carriers. For example, golgin-97, but not p230, is associated with distinct membrane extensions of the TGN loaded with E-cadherin and knockdown of golgin-97 selectively blocked exit of E-cadherin cargo from the TGN (21). Collectively, our studies have shown that golgin-97 and p230 are essential components of distinct sets of tubulovesiclar carriers that emerge from the TGN. Because the post-Golgi exocytic trafficking of both TNF (5) and E-cadherin (23) involves transport via the recycling endosome to the cell surface, the TGN golgins are likely

to be regulators of transport from the TGN to the recycling endosome.

A key question remains as to how p230 regulates exit of TNF from the Golgi. Live imaging indicated that the total number of tubulation and budding events for p230 and TNF is roughly similar (see Fig. 2 and SI Movies 1 and 2). However, more TNF-loaded carriers (≈75/cells per minute/100 μm^2) were observed than p230-labeled carriers (≈35/cells per minute/100 μm^2), indicating that p230 is typically retained at the TGN rather than being actively sorted into detaching carriers containing TNF. This finding implies that p230 might be crucial for the sorting/segregation of TNF but is not likely to drive scission events. This is supported by our previous observation that p230 is locally depleted from intratubule membrane domains that subsequently undergo membrane scission, implying that p230 is displaced by local concentrations of membrane fission machinery (21). Furthermore, given the observation that the TNF cargo is typically localized to the distal tips of tubules emerging from the TGN, it is likely that the tip of the extending tubules contains a complex of proteins including cargo association/sorting machinery, scission machinery, and motor proteins (to motivate tubule extension).

Although this work represents a demonstration of a physiologically regulated role for p230 in mammalian cell protein transport, tantalizing hints of this mechanism have been revealed in both yeast and the protozoan parasites. For example in *Trypanosoma brucei*, Arl (an upstream regulator of p230) is essential for the up-regulated transport of a GPI-anchored protein (26), suggesting an evolutionarily conserved role for p230 in lipid raft-oriented Golgi to plasma membrane trafficking.

We have demonstrated the efficacy of miRNA based vectors for silencing TGN golgins in cultured cells (24, 27), and this study demonstrates the potential of miRNA-based vectors for silencing in transgenic mice. The use of a miRNA-based vector system, with an RNA pol II-regulated promoter, has an advantage over other RNAi systems in allowing the multicistronic cotranscription of the reporter gene GFP and therefore the tracking of miRNA production in individual cells. Another advantage is the potential for tissue-specific expression of the miRNA-silencing system. It is important to note that the miRNA constructs used here do not appear to perturb normal developmental pathways, because the reconstitution of mice with the p230 miRNA bone marrow stem cells did not influence T and B cell repopulation (results not shown), implying that the intracellular levels of miRNA are not saturating the endogenous small RNA pathways. Hence, this RNAi strategy has the potential to dissect different transport pathways in a range of specialized cell types. The demonstration here of the ability to block the secretion of TNF *in vivo* has potential for treatment of a variety of inflammatory conditions. More broadly, the control of cytokine secretion by the specific silencing of trafficking machinery has potential application in modulating a range of immune responses.

Materials and Methods

Plasmids, Antibodies, and Reagents. Constructs encoding GFP-TNF, YFP-TNF, human E-cadherin-GFP (Ecad-GFP), myc-tagged full length p230, YFP-tagged C-terminal GRIP domains of p230/golgin-245 (YFP-p230GRIP), and golgin-97 (YFP-golgin-97-GRIP) have been described (11, 21). To generate retroviral DNA constructs expressing microRNA (miRNA), miRNA inserts from recombinant pcDNA 6.2GW/EmGFP expression vectors (Invitrogen) were amplified and then subcloned into pMIG-MSCV vector (28) to produce pMIG-MSCV constructs. The following primary antibodies were used: human autoantibodies to p230 and affinity-purified rabbit polyclonal antibodies to p230 (8), rabbit polyclonal antibodies to human GCC88 and GCC185 (13), mouse monoclonal antibody to golgin-97 (BD Biosciences), mouse monoclonal anti-α-tubulin (Amersham), rabbit polyclonal antibody to mouse TNF (Chemicon), 9E10 mouse monoclonal antibody specific for the myc epitope (29), and mouse monoclonal antibody to human E cadherin (HECD1) (M. Takeichi, Kyoto University, Kyoto). Murine MHC class II was detected by using anti-I-E antibodies (clone 14–4-4S). LPS from *Escherichia coli* 011:B4 was purchased from Sigma Aldrich. TACE inhibitor, TAPI-1, was purchased from Calbiochem. Secondary antibodies used for immunofluorescence were goat anti-rabbit IgG-Alexa Fluor 568, goat anti-rabbit IgG-Alexa Fluor 488, goat anti-human Alexa Fluro 647, and goat anti-human Alexa Fluor 594 were from Molecular Probes and goat anti-rabbit IgG–Cy5 was from Amersham. Horseradish peroxidase-conjugated sheep anti-rabbit Ig and anti-mouse Ig were from Dako.

Cell Culture and Transfection. HeLa cells and 3T3 mouse fibroblasts were maintained as semiconfluent monolayer in DMEM supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine, 100 units/μl penicillin, and 0.1% (wt/vol) streptomycin (C-DMEM). RAW264.7 murine macrophages were cultured as described (30). For transfections, HeLa cells and 3T3 mouse fibroblasts were seeded as monolayers and transfected by using Fugene 6 (Roche Diagnostics), according to the manufacturer's instructions. Transfections were carried out in C-DMEM at 37°C, 10% CO_2 for 24–96 h. Transient transfection of siRNA was performed by using Oligofectamine (Invitrogen). RAW 264.7 murine macrophages were transfected with miRNA constructs either by electroporation or by using Lipofectamine 2000 (Invitrogen), as described (4).

RNA Interference (siRNA and miRNA). Mouse- and human p230-specific siRNA duplex (22) and human GCC88-specific siRNA duplex (27) have been described. For knockdown of mouse p230 and GCC185 using a miRNA system (Invitrogen), primer sets were designed by using Invitrogen BLOCK-iT RNAi designer, annealed, and cloned into pcDNA 6.2GW/EmGFP miR expression vector containing a GFP expression cassette. See *SI Materials and Methods* for details of primers.

Assay for TNF Secretion by Activated Macrophages. The trafficking of TNF from the Golgi to the cell surface was measured as described (5). To detect cell-surface TNF, activated macrophages were stimulated as described above in the presence of 10 μM TAPI-1, fixed, and nonpermeabilized cells stained for surface TNF. Flow cytometric analyses were performed on a FACStar (BD Biosciences) by using CellQuest Pro.

Generation of p230-Depleted Transgenic (Retrogenic) Mice. Retroviral producer cell lines were generated as described (28, 31). The protocol for the retroviral-mediated stem cell gene transfer has also been described (32). See *SI Materials and Methods* for details.

Isolation of Peritoneal Macrophages, Immunofluorescence, Immunoblotting, and Live-Cell Imaging and Quantitation. See *SI Materials and Methods* for detailed experimental procedures for isolation of peritoneal macrophages, immunofluorescence, immunoblotting, and live-cell imaging and quantitation.

ACKNOWLEDGMENTS. This work was supported by funding from the Australian Research Council (P.A.G.) and the Australian National Health and Medical Research Council (J.L.S.). Z.Z.L. is supported by Australian Postgraduate Awards, and N.L.G. is supported by a National Health and Medical Research Council R. D. Wright Career Development Award and by project grants.

1. Hume DA (2006) The mononuclear phagocyte system. *Curr Opin Immunol* 18:49–53.
2. Beutler BA (1999) The role of tumor necrosis factor in health and disease. *J Rheumatol* 57:16–21.
3. Siddiqui MA, Scott LJ (2005) Infliximab: a review of its use in Crohn's disease and rheumatoid arthritis. *Drugs* 65:2179–2208.
4. Manderson AP, Kay JG, Hammond LA, Brown DL, Stow JL (2007) Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. *J Cell Biol* 178:57–69.
5. Murray RZ, Kay JG, Sangermani DG, Stow JL (2005) A role for the phagosome in cytokine secretion. *Science* 310:1492–1495.
6. Murray RZ, Wylie FG, Khromykh T, Hume DA, Stow JL (2005) Syntaxin 6 and Vti1b form a novel SNARE complex, which is up-regulated in activated macrophages to facilitate exocytosis of tumor necrosis factor-alpha. *J Biol Chem* 280:10478–10483.
7. Gleeson PA, Lock JG, Luke MR, Stow JL (2004) Domains of the TGN: Coats, tethers and G proteins. *Traffic* 5:315–326.
8. Kooy J, Toh BH, Pettitt JM, Erlich R, Gleeson PA (1992) Human autoantibodies as reagents to conserved Golgi components - Characterization of a peripheral, 230-kDa compartment-specific Golgi protein. *J Biol Chem* 267:20255–20263.
9. Fritzler MJ, Lung CC, Hamel JC, Griffith KJ, Chan EKL (1995) Molecular characterization of golgin-245, a novel Golgi complex protein containing a granin signature. *J Biol Chem* 270:31262–31268.
10. Erlich R, Gleeson PA, Campbell P, Dietzsch E, Toh BH (1996) Molecular characterization of transGolgi p230 - A human peripheral membrane protein encoded by a gene on chromosome 6p12–22 contains extensive coiled-coil alpha-helical domains and a granin motif. *J Biol Chem* 271:8328–8337.

11. Gleeson PA, *et al*. (1996) p230 is associated with vesicles budding from the trans-Golgi network. *J Cell Sci* 109:2811–2821.
12. Griffith KJ, *et al*. (1997) Molecular cloning of a novel 97-kD golgi complex autoantigen associated with Sjogrens-syndrome. *Arthritis Rheum* 40:1693–1702.
13. Luke MR, Kjer-Nielsen L, Brown DL, Stow JL, Gleeson PA (2003) GRIP domain-mediated targeting of two new coiled-coil proteins, GCC88 and GCC185, to subcompartments of the trans-Golgi network. *J Biol Chem* 278:4216–4226.
14. Barr FA (1999) A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. *Curr Biol* 9:381–384.
15. Kjer-Nielsen L, Teasdale RD, van Vliet C, Gleeson PA (1999) A novel Golgi-localisation domain shared by a class of coiled-coil peripheral membrane proteins. *Curr Biol* 9:385–388.
16. Munro S, Nichols BJ (1999) The GRIP domain- a novel Golgi-targeting domain found in several coiled-coil proteins. *Curr Biol* 9:377–380.
17. Panic B, Whyte JR, Munro S (2003) The ARF-like GTPases Arl1p and Arl3p act in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Curr Biol* 13:405–410.
18. Gangi Setty SR, Shin ME, Yoshino A, Marks MS, Burd CG (2003) Golgi recruitment of GRIP domain proteins by Arf-like GTPase 1 is regulated by Arf-like GTPase 3. *Curr Biol* 13:401–404.
19. Lu L, Hong W (2003) Interaction of Arl1-GTP with GRIP domains recruits autoantigens Golgin-97 and Golgin-245/p230 onto the Golgi. *Mol Biol Cell* 14:3767–3781.
20. Jackson CL (2003) Membrane traffic: Arl GTPases get a GRIP on the Golgi. *Curr Biol* 13:R174–R176.
21. Lock JG, Hammond LA, Houghton F, Gleeson PA, Stow JL (2005) E-cadherin transport from the trans-Golgi network in tubulovesicular carriers is selectively regulated by golgin-97. *Traffic* 6:1142–1156.
22. Derby MC, *et al*. (2007) The trans-Golgi Network Golgin, GCC185, is Required for Endosome-to-Golgi Transport and Maintenance of Golgi Structure. *Traffic* 8:758–773.
23. Lock JG, Stow JL (2005) Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol Biol Cell* 16:1744–1755.
24. Derby MC, Gleeson PA (2007) New insights into membrane trafficking and protein sorting. *Int Rev Cytol* 261:47–116.
25. Panic B, Perisic O, Veprintsev DB, Williams RL, Munro S (2003) Structural basis for Arl1-dependent targeting of homodimeric GRIP domains to the Golgi apparatus. *Mol Cell* 12:863–874.
26. Price HP, Panethymitaki C, Goulding D, Smith DF (2005) Functional analysis of TbARL1, an N-myristoylated Golgi protein essential for viability in bloodstream trypanosomes. *J Cell Sci* 118:831–841.
27. Lieu ZZ, *et al.* (2007) The Golgin, GCC88, Is Required for Efficient Retrograde Transport of Cargo from the Early Endosomes to the Trans-Golgi Network. *Mol Biol Cell* 18:4979–4991.
28. Szymczak AL, *et al*. (2004) Correction of multi-gene deficiency *in vivo* using a single "self-cleaving" 2A peptide-based retroviral vector. *Nat Biotechnol* 22:589–594.
29. Evan GI, Lewis GK, Ramsay G, Bishop JM (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* 5:3610–3616.
30. Shurety W, Merino-Trigo A, Brown D, Hume DA, Stow JL (2000) Localization and post-Golgi trafficking of tumor necrosis factor-alpha in macrophages. *J Interferon Cytokine Res* 20:427–438.
31. Holst J, *et al*. (2006) Generation of T-cell receptor retrogenic mice. *Nat Protocols* 1:406–417.
32. Persons DA, *et al*. (1997) Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors *in vitro* and identification of genetically modified cells *in vivo*. *Blood* 90:1777–1786.