Tubulin- and actin-associating GIMAP4 is required for IFN-γ secretion during Th cell differentiation

Mirkka T Heinonen1,2,3, Kartiek Kanduri2, Harri J Lähdesmäki4, Riitta Lahesmaa2,5 and Tiina A Henttinen1,5

Although GTPase of the immunity-associated protein (GIMAP) family are known to be most highly expressed in the cells of the immune system, their function and role remain still poorly characterized. Small GTPases in general are known to be involved in many cellular processes in a cell type-specific manner and to contribute to specific differentiation processes. Among GIMAP family, GIMAP4 is the only member reported to have true GTPase activity, and its transcription is found to be differentially regulated during early human CD4+ T helper (Th) lymphocyte differentiation. GIMAP4 has been previously connected mainly with T- and B-cell development and survival and T-cell apoptosis. Here we show GIMAP4 to be localized into cytoskeletal elements and with the component of the trans golgi network, which suggests it to have a function in cellular transport processes. We demonstrate that depletion of GIMAP4 with RNAi results in downregulation of endoplasmic reticulum localizing chaperone VMA21. Most importantly, we discovered that GIMAP4 regulates secretion of cytokines in early differentiating human CD4+ Th lymphocytes and in particular the secretion of interferon-γ also affecting its downstream targets.

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The main response of an innate immune cell towards infection and inflammation is the production and release of cytokines. The cytokines pass through multiple cell compartments by complex and tightly regulated transport processes. The specific route of transportation depends on the cargo, but in general the secreted cytokines are delivered via the lumen of the endoplasmic reticulum (ER), prior to entering the trans golgi network (TGN), from which they are transported to their final destination. Malfunction of any of the components in these pathways leads to changes in the secretory process and thus in cell function. One group of proteins known to function in vesicular trafficking are the small GTP-binding proteins. Over 60 mammalian Rab GTPases are known to be associated with cell compartment maintenance and specification.1–3

The conserved GTPase of the immunity-associated protein (GIMAP) family has been described as a new class of putative small GTPases involved in the defense systems among vertebrata and angiosperm plants.1–4 The human GIMAP family consists of eight genes, seven of which are expressed as functional proteins. The entire GIMAP family has been found to be highly expressed in the cells of the immune system (reviewed by Filen and Lahesmaa5) and closely associated with T-cell differentiation and B- and T-cell development.6–13 Additionally, GIMAP proteins have been connected with cancer and immune-mediated diseases.14–18 Structurally, GIMAP proteins are classified to the TRAFAC (translation factor associated) class of GTPases.17,18 In a recent study, Schwefel et al.18 suggested that GIMAP2 shares structural similarities with the dynamin, Toc and septin GTPases. Additionally, they proposed on the basis of structural similarity that the entire GIMAP family could share functions similar to those of septins. Septins contribute to intracellular transport, cell migration and cell division and have been predicted to act as scaffolding proteins bringing together other regulatory and signalling proteins.19 Most members of the human GIMAP family have a GTPase domain, and all family members have domains involved in protein–protein interactions.1–20 Many are localized in cellular organelles involved in transport processes, such as ER and Golgi.1,20

Although the GIMAP proteins have been the focus of a number of recent studies, their exact function and role in immune response remains to be clarified. It is likely that GIMAPs participate in a variety of cellular processes of the immune system, and there is increasing evidence suggesting their role in transport and secretion. Chen et al.21 found that GIMAP5 partially co-localized with tubulin and that the GIMAP5-mediated increase in mitochondrial Ca2+ to be dependent on the microtubules and actin cytoskeleton. Calcium signalling and cytoskeletal changes are also important during the T-cell receptor (TCR) activation.22 Using automated microscopy-based genome-wide RNAi screens in cultured human cells, Simpson et al.23 demonstrated that downregulation of GIMAP2 and GIMAP6 interfered with ER-to-plasma membrane transport of the secretory cargo membrane protein tsO45G. Pascall et al.24 found evidence connecting GIMAP6 with autophagy, and Weiss et al.25 suggested that the septin-like properties

1Division of Genetics and Physiology, Department of Biology, Laboratory of Animal Physiology, University of Turku, Turku, Finland;
2Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland;
3Turku Doctoral Programme of Molecular Medicine, TuDMM and Turku Doctoral Programme of Biomedical Sciences, TuBS Turku, Finland and
4Department of Information and Computer Science, Aalto University Helsinki, Finland

*These authors contributed equally to this work.
Correspondence: Professor R Lahesmaa, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Tykistökatu 6A, Turku FIN-20520, Finland.
E-mail: ritta.lahesmaa@tkk.fi
or Adj. Professor TA Henttinen, Division of Genetics and Physiology, Department of Biology, Laboratory of Animal Physiology, University of Turku, Turku FI-20014, Finland.
E-mail: tiina.henttinen@utu.fi

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of the GIMAP proteins endorse their role in membrane trafficking at phagolysosomal membranes or in constraining and compartmentalizing pathogens within the coral cell. Jokinen et al.26,27 speculated that GIMAP3 could have a role in either the formation or fusion of the mitochondria-driven vesicles.

Compared with the other members of the family, GIMAP4 has two unique characteristics that make it an interesting target in the context of Th cell studies: it has GTPase activity on its own28 and a calmodulin-binding IQ domain, which is known to be involved in calcium signalling.29,30 We have previously shown differential regulation of GIMAP4 during the early stages of human T helper (Th) cell differentiation, with upregulation and downregulation observed under Th1- and Th2-promoting condition, respectively.6 Many of the previous studies of GIMAP4 have concentrated on its function in murine T-cell survival and revealed association with the proapoptotic Bax protein.8,29,31 GIMAP4 role regarding TCR signalling and secretory pathways has not been addressed.

To study the role of GIMAP4 in human lymphocytes, we examined its localization in human CD4+ Th cells, with a supporting localization measurements in HeLa cells. We show GIMAP4 to be associated with cytoskeletal microtubules, actin and with TGN. Interestingly, analysis through GIMAP4 depletion combined with genome-wide transcriptomics revealed that GIMAP4 regulates ER-localized chaperone, Vacuolar ATPase assembly integral membrane protein VMA21. Moreover, the depletion of GIMAP4 did not change the TCR activation-induced changes in transcriptional gene expression (as measured by microarrays (data not shown). These findings emphasize the function of GIMAP4 in transportation processes orchestrated by the ER–Golgi network. Most importantly, we found that depletion of GIMAP4 results in decreased interferon (IFN)-γ secretion, leading to disruption of the IFN-γ-regulated pathway and the downstream IFN-γ signalling, which is important in driving and maintaining the Th1 cell phenotype.

RESULTS

GIMAP4 interacts with microtubules and TGN

GIMAP4 has been earlier localized to the water soluble25 and microsomal6 fractions of the cytoplasm, ER and Golgi.1 GIMAPs are related to septin GTPases known to interact with microtubules and membranes.18 Thus, we wanted to explore whether GIMAP4 associates with actin and β-tubulin. The confocal imaging results show the assembly of GIMAP4 into filament-like structures and partially co-localized with actin and β-tubulin in HeLa cells (Figures 1a and b). As the microtubule network is vital for endosome trafficking, we investigated the localization of GIMAP4 with known markers for different compartments of vesicle transport system. We found GIMAP4 to co-distribute with syntaxin-6, which localizes and functions in TGN32 (Figure1c). GIMAP4 did not localize in the same cellular compartment as lysosomal marker LAMP1 (Figure1d) and neither co-distribution nor co-localization of GIMAP4 with early endosomal marker EEA1 (Figure1e).

Figure 1 GIMAP4 interacts with microtubule and TGNs in HeLa cells and co-localizes with known vesicular transport markers in CD4+ cells induced to Th1 direction. HeLa cells growing on the glass coverslips were fixed and probed with antibodies against GIMAP4, EEA1, Rab27A, syntaxin-6 or LAMP1-Cy3. CD4+ T cells activated by anti-CD3/anti-CD28 were cultured in the presence of IL-12 for 4 days, fixed and probed with antibodies against GIMAP4, EEA1, Rab27A, syntaxin-6 or LAMP1-Cy3. Secondary antibodies used were conjugated with Alexa Fluor-488 or -546. Washed cells were mounted on Vectashield HardSet mounting medium with 4,6-diamidino-2-phenylindole, and confocal images were acquired on a Zeiss LSM 510 laser-scanning confocal microscope. GIMAP4 is assembled into filament-like structures spatially co-localized with actin (a), β-tubulin (b) and syntaxin-6 (c). The white colour indicates co-localization of two labelled antigens (R = Mander’s Overlap coefficient). There is a co-distribution but no co-localization of GIMAP4 with lysosomal marker LAMP1 (d) and neither co-distribution nor co-localization of GIMAP4 with early endosomal marker EEA1 (d). In T cells, the GIMAP4 localization is spatially asymmetric (e, left) in contrast to naive CD4+ T cells where punctate staining of GIMAP4 is seen throughout the cytoplasm (e, right). The endogenous markers used for the endosomal compartments, LAMP1 and syntaxin-6, co-distribute with GIMAP4, whereas EEA1 or Rab27A do not (f). The white lines denote the linear fluorescence profile analysis line shown in the graphs.
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compartment as lysosomal marker LAMP1 or with early endosomal marker EEA1 (Figure 1d), suggesting that GIMAP4 is not involved in the endocytic processes. The results indicated that GIMAP4 could interact both with microtubule network and TGN in HeLa cells.

To investigate the localization of GIMAP4 in corresponding Th cell organelles, we examined its localization in the endosomal vesicle pathway: CD4+ T cells were activated with anti-CD3/anti-CD28, and Th1 polarization was induced by culturing the cells for 3–4 days with interleukin (IL)-12. After 4–5 days of culture, GIMAP4 was found to be spatially asymmetric in most of the activated cells (Figure 1e, left). In contrast, punctate staining of GIMAP4 was seen throughout the cytoplasm of the naive cells (Figure 1e, right). Of the markers used to study GIMAP4 localization in activated cells, only LAMP1 and syntaxin-6 were located in the same cell compartment as GIMAP4, whereas EEA1 or Rab27A did not appear to be significantly co-distributed with GIMAP4 (Figure 1f). In agreement with the results from HeLa cells, GIMAP4 was also observed to localize in the TGN cell compartment of the Th cell. Taken together, these results support GIMAP4 function in secretory processes.

GIMAP4 does not influence TCR activation-induced regulation of gene transcription but regulates the expression of ER-localizing chaperone VMA21

Genome-wide transcriptomic profiling was conducted to investigate the effects of GIMAP4 depletion in CD4+ T cells during TCR activation. Cord blood (derived) CD4+ T cells were nucleofected with either GIMAP4 gene-specific (siGimap4) or non-targeting (siScramble) siRNA. The samples for microarray analysis from three biological replicates were collected at 43 h after nucleofection. The GIMAP4 downregulation was confirmed by western blotting (data not shown). The statistical analyses revealed one gene, VMA21, to be differentially expressed before Th cell activation (logFC = −1.3, adj. P-value = 0.0035, data not shown). The effect was further corroborated by western blotting for VMA21 expression from two biological replicates (Figure 2). GIMAP4 did not, however, influence the expression of any of the immediate- or early activation-induced genes, as the results from the samples collected 2 h after anti-CD3/anti-CD28 TCR activation did not reveal any differentially expressed genes. Additionally, the surface expression of CD69 T-cell activation marker was detected at 6 h and 24 h in siGimap4-treated cells compared with the non-targeting siScramble-treated controls. The results showed that downregulation of GIMAP4 affected the secretion of all cytokines measured (Supplementary Figure S1).

Of the cytokines measured, IFN-γ is a marker cytokine of Th1 cell lineage and the most prominent cytokine secreted in response to TCR activation. Under the conditions of GIMAP4 downregulation, the IFN-γ concentration in the culture supernatant was decreased at all the time points measured (Supplementary Figure S1). The measurements were repeated for IFN-γ in two additional biological replicates from samples collected on the first and the third day of the culture, and the mRNA expression was also determined up until the fourth day of culture. In response to downregulation of GIMAP4 expression, the ability of Th cells to secrete IFN-γ was reduced at 24 h (Figure 3a, P = 0.060) and more so at 72h (Figure 3b, P = 0.013). The downregulation of GIMAP4 did not affect the amount of intracellular IFN-γ at 24, 48 and 96 h after initial activation (n = 3), as shown in Figure 3c. IFN-γ mRNA expression was only affected at 96 h after initial activation (Figure 3d, 96 h P = 0.018). These data suggest that IFN-γ secretion and the consequent IFN-γ autoregulatory signalling are impaired in GIMAP4-deficient cells. The data from multiple replicates are presented as an average expression with s.d.

GIMAP4 regulates IFN-γ signalling pathway in primary Th1 cells

Next, we asked whether the decreased IFN-γ secretion following downregulation of GIMAP4 results in disruption of the IFN-γ-induced Janus-activated kinase/signal transducer and activator of transcription factor (JAK/STAT) signalling pathway. Compared with controls, downregulation of GIMAP4 resulted in the decrease of total STAT1 and pSTAT1 expression in anti-CD3/anti-CD28 activated CD4+ T cells, cultured under Th1-driving conditions for 24 h. As expected, decrease in IFN-γ secretion after downregulation of GIMAP4 is reflected in the activation of downstream targets of IFN-γ signalling (Figure 4a, P = 0.049 for pSTAT1, P = 0.017 for STAT1 and P = 0.015 pSTAT1/STAT1, n = 3). To further prove that decreased STAT1 expression and phosphorylation is a result of a decreased level of IFN-γ in the cell supernatant rather than malfunction of IFN-γ receptor, we added IFN-γ to the GIMAP4-depleted cells. CD4+ T cells were nucleofected with siGimap4 and siScramble siRNAs, and the cells were activated by anti-CD3/anti-CD28. The cells were induced to differentiate into Th1 direction by adding IL-12 with or without extracellular IFN-γ (100 U). Samples were collected for analysis 96 h after initial activation. The results from the two biological replicates (Figure 4b) indicated that addition of IFN-γ to the GIMAP4-
deficient cells restores the STAT1 expression and phosphorylation, as expected. We studied the IFN-γ pathway further downstream by measuring the expression of two different Th1-specific cell surface receptors, STAT-1-regulated tyrosine-kinase receptor IL12Rβ2 and the nuclear factor-kB-regulated G-protein coupled receptor C-X-C motif chemokine receptor 3 (CXC3). The representative results from three biological replicates are shown in Figure 4c. The expression of CXC3 compared with the matching controls show no significant changes under GIMAP4 downregulation in anti-CD3/anti-CD28-activated CD4+ T cells, cultured under Th1-inducing conditions for 24, 48 and 72 h after initial activation. Depletion of GIMAP4, however, downregulated the expression of IL12Rβ2 at 72 h (two-tailed t-test \( P = 0.034 \)) after initial activation and additionally at 48 h (two-tailed t-test \( P = 0.003 \)) when the cells were cultured without IL-12. The expression of IL12Rβ2 mRNA was also decreased at 48 h in GIMAP4-depleted cells cultured in Th1-induced conditions (two-tailed t-test \( P = 0.022 \)). These results suggest that depletion of GIMAP4 under Th1 conditions has a long-lasting effect on the IFN-γ receptor-mediated downstream signalling but not to the receptor function itself. The data from multiple replicates are presented as an average expression with s.d.

GIMAP4 depletion does not compromise cell viability of primary human CD4+ T cells during early differentiation

Septins are known to form microfilament structures with each other or with filamentous proteins, such as cytoskeletal actin, myosin and tubulin, and have a functional role in cell division among many other functions (reviewed by Peterson and Petty33). The septin-like nature of GIMAP proteins18 and GIMAP4 co-localization with microtubules together with the reported GIMAP4 association with T-cell survival led us to investigate GIMAP4 influence in cell cycle, proliferation and apoptosis of early differentiating human CD4+ Th cells. This was important in order to rule out the possibility that an increase in apoptosis or disruption of cell cycle could, in part, explain the observed decrease in IFN-γ secretion in response to GIMAP4 downregulation. To study the cell cycle, synthetic 5-bromo-2-deoxyuridine (BrdU) was added to the siGimap4- and siScramble-treated cell cultures according to the manufacturer’s instructions at the point of activation and Th1 induction. The cells were stained at 48 and 72 h with anti-BrdU-FITC (fluorescein isothiocyanate) and 7-aminoactinomycin D, and the fluorescence was measured with flow cytometry. The uptake of BrdU (Figure 5a) was similar in siGimap4 cells and in siScramble-treated cells (48 h \( P = 0.074 \)). These results indicated that downregulation of GIMAP4 does not disturb the cell cycle. Moreover, depletion of GIMAP4 did not influence proliferation, as measured by carboxyfluorescein succinimidyl ester staining (data not shown). Finally, the effect of GIMAP4 downregulation on T-cell apoptosis was measured by detecting active caspase 3 from siGimap4- and non-targeting siScramble-treated cells before and 24 h after activation and addition of IL-12 (Figure 5b). The gating of the cell populations for the active Caspase 3 was done according to the unstained control sample (not shown) and for the dead cell counts according to the siScramble sample. The results from three biological replicates showed no statistically significant effect between the siGimap4-treated cells compared with the controls in the levels of active caspase 3 or the amount of dead cells. Additionally, no significant differences were observed for the corresponding Annexin V and PI measurements (data not shown). Overall these results indicated that GIMAP4 deficiency did not affect the cell cycle or caspase-mediated T-cell apoptosis in the system at the time points studied. The data from multiple replicates is presented as an average expression with s.d.
DISCUSSION

GTPases regulate several cellular processes in response to a variety of external stimuli (reviewed in Scheele et al.34). The GIMAP family is a newly described class of putative GTPases distinct from other small GTPases, whose function is mainly connected to the immune system. In the light of the earlier studies, which indicated the involvement of GIMAP in TCR signalling, as well as secretory and transport processes,18,19,21,23–25,27 we addressed the role of GIMAP4 in early phase of Th cell differentiation. First, using a panel of antibodies for molecules engaged in secretory trafficking, we visualized the cellular compartments in which GIMAP4 is localized. From these measurements, the observed subcellular positioning of GIMAP4 was in line with previous structural studies.18 These results suggest the involvement of GIMAP4 in the constitutive secretory processes that lymphocytes use to launch the expression and secretion of cytokines in response to external stimuli. The co-localization with syntaxin-6 suggests that GIMAP4 has a role in the TGN. The lack of co-localization with Rab27, in turn, excludes GIMAP4 from the later transport processes, such as granule exocytosis or vesicle docking and fusion.

In line with the observations from the localization experiments, genome-wide transcriptional profiling of GIMAP4-depleted cells further supported the role of GIMAP4 in the ER–Golgi function and vesicle trafficking during the early Th cell differentiation. Here we demonstrated that GIMAP4 regulates the expression of the ER-associated protein VMA21. In yeast, VMA21 has been shown to function as an ER-localizing chaperone that assembles the V-ATPase proton pump, which in turn is a global ATPase in membranes and in secretory vesicles.35 Another study in yeast indicates that VMA21 function is related to the vesicular structures and the trafficking between ER and Golgi.36 The function and regulation of VMA21 in human Th cells, as well as the role of GIMAP4 in this context, remains to be further studied. Nonetheless, the observed localization and differential regulation of VMA21 detected in this study, together with previous findings regarding related GIMAP family members, support
Figure 5 Downregulation of GIMAP4 does not influence the cell cycle of primary human CD4+ T cells. At the time of activation and Th1 induction, synthetic thymidine analogue BrdU was added to the siGimap4- and siScramble-treated cultures according to the manufacturer’s instruction. The samples were collected at 48 h (two-tailed t-test \(P=0.074\)) and 72 h from activation. The samples were stained with anti-BrdU-FITC, and the BrdU signal was measured with a flow cytometer. After the exclusion of apoptotic cells, the number of cells which had incorporated BrdU (R2) was determined from three individual experiments (a). Data are presented as an average expression with s.d. The active caspase 3 and the number of dead cells were detected from siGimap4- and non-targeting siScramble-treated cells before activation and 24 h after initial activation and addition of IL-12. Representative results from the three biological replicates is shown (b). Gating for active caspase 3 was done according to the siScramble sample (not shown).
the involvement of GIMAP4 in cellular transport processes associated with the ER and Golgi.

On the basis of observations concerning in the abundance of GIMAP4 in Th1 cell differentiation,6 and the suggested role for microtubules in the targeted secretion of IFN-γ,37 we resolved to determine how GIMAP4 depletion influenced cytokine, and specifically IFN-γ, secretion in the early stages of human Th1 cell differentiation. As the downregulation of GIMAP4 significantly decreased IFN-γ secretion, we further studied the role of GIMAP4 in the signalling cascade and the Th1 differentiation process by using STAT1, IL12Rβ2 and CXCR3 as readouts. During canonical Th1 differentiation, IFN-γ expression is upregulated and maintained by JAK–STAT1 signalling pathways by autoregulation through STAT1.38,39 IFN-γ regulated IL-12 signalling drives Th1 differentiation, whereas CXCR3 contributes to the migration of Th1 cells. Our results indicated that depletion of GIMAP4 causes a decrease in IFN-γ secretion that was observed throughout the course time studied, and later also a decrease in the expression of the IFN-γ gene was observed. The late effect on gene expression is most probably due to the decreased IFN-γ, which then influences the IFN-γ autoregulatory feedback loop.40 Consistent with the reduced IFN-γ secretion and production, STAT1 phosphorylation was also decreased. IL12Rβ2 expression, which is also known to be maintained by IFN-γ signalling,41,42 was slightly affected at the third day of differentiation, but the CXCR3 expression was not. In addition, we verified that the level of intracellular IFN-γ was not affected by GIMAP4 depletion, and thus excluded the notion that the low amount of extracellular IFN-γ could be a result from posttranscriptional changes in the intracellular process.

Taken together, our results indicated that the affect of GIMAP4 depletion on the downstream signalling cascade resulted in reduced IFN-γ secretion and not from a defect in the functionality of IFN-γ receptor itself or the downstream signalling components. The IFN-γ/STAT1 signalling cascade was restored by exogenous IFN-γ, thus showing that the amount of extracellular IFN-γ is the primary source of this effect. Although earlier studies report that GIMAP proteins are involved in cell cycle, cell proliferation and/or survival,10,29,43,44 the observation whether the GIMAP proteins are antiapoptotic or proapoptotic varies between the rat and mouse studies.8,45–47 As the influence of GIMAP4 in apoptosis and cell survival was not known in our experimental set up, we thus examined whether the decreased IFN-γ secretion could be caused by changes in apoptosis, cell cycle and/or proliferation of the GIMAP4-depleted, differentiating Th1 cells. As there were no significant changes detected, we thus concluded that GIMAP4’s role in secretory processes during the early differentiation of naive human Th1 cells is not biased by apoptosis. Interestingly, this result is not fully in line with the earlier study by Schnell et al.29 in which Gimap4 was observed to influence T-cell apoptosis. This discrepancy can be explained by the difference in study settings. We have examined the effect during the early differentiation of anti-CD3/anti-CD28-activated, Th1-induced human CD4+ T cells (without apoptosis induction), whereas the observations of Schnell et al.29 were made from the mouse spleen cells under apoptosis-inducing conditions.

In conclusion, our results demonstrate that human GIMAP4, associates with intracellular membrane structures and co-localizes with the cytoskeletal actin and β-tubulin, and with the TGN. Our experiments show that depletion of GIMAP4 does not affect the TCR activation-induced transcription, but does impair IFN-γ secretion and the subsequent IFN-γ signalling cascade, and affects the IL12Rβ2 expression. Together these observations support the role of GIMAP4 in processes involved in the secretory cargo transport. Furthermore, GIMAP4 regulates ER-localizing chaperone VMA21, which has been linked to vesicular trafficking, thus providing additional evidence of the role that GIMAP4 has in TGN and ER-orchestrated transport pathways and its influence in Th cell differentiation processes.

METHODS

Cell culture

HeLa cells (ATCC) were cultured in Dulbecco’s Modified Eagle’s Medium (Thermo Fisher, Invitrogen, Waltham, MA, USA) supplemented with penicillin and streptomycin (50 μg ml⁻¹), 2 mM l-glutamine and 10% fetal calf serum. For immunostainings, cells were cultured on glass cover slips (0.5×10⁴ cell ml⁻¹). Mononuclear cells were isolated by Ficoll-Paque (GE Healthcare, Little Chalfont, UK) gradient centrifugation from umbilical cord blood samples collected from healthy neonates at Turku University Hospital. CD4+ T cells were enriched by anti-CD4-conjugated magnetic beads (Dynal Beads, Thermo Fisher, Invitrogen) and cultured in Yssel’s medium supplemented with 1% AB serum (Red Cross Finland Blood service, Helsinki, Finland). Cells were activated with 2.5 μg ml⁻¹ plate-bound anti-CD3 and with 500 ng ml⁻¹ soluble anti-CD28 (Immunotech, Vaudreuil-Dorion, Quebec, Canada) in a density of 1.7–2×10⁶ cells ml⁻¹. To induce Th1 differentiation, 2.5 ng ml⁻¹ IL-12 (R&D Systems, Minneapolis, MN, USA) was added to the culture medium at the time of activation. On the second day of culture, IL-2 (17 ng ml⁻¹, R&D Systems) was added. In the rescue experiment, 100 U of IFN-γ (Abcam, Cambridge, UK, ab19140) was added to the culture. All cell cultures consisted of a pool of CD4+ T cells isolated from at least five donors. The use of human blood from unknown donors was permitted by the Finnish Ethics Committee.

Confocal microscopy

The harvested non-activated or anti-CD3/anti-CD28 activated (as described in ‘Cell culture’ section) and IL-12-treated CD4+ T cells were cultured for 4 days, and HeLa cells growing on the glass cover slips were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 min on ice. The cells were permeabilized with 0.2% saponin for 10 min, blocked with 10% fetal bovine serum and probed with antibodies against rabbit α-GIMAP4-antiserum (a gift from Dr Cambot) or β-tubulin (Sigma-Aldrich, St Louis, MO, USA), EEA1, Rab27A, syntaxin-6 and LAMP1-Cy3 (Abcam) and Alexa Fluor-488- or -546-conjugated secondary antibodies (Thermo Fisher, Life Technologies, Waltham, MA, USA). Alexa Fluor-546 phalloidin (Thermo Fisher, Invitrogen) was used to stain F-actin. Cells were washed and mounted on Vectashield HardSet medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Confocal images were acquired on a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany). The linear fluorescence profile analysis was made with the ZEN lite software (Zeiss) across the white line indicated in the figure. BioimageXD program (BioimageXD, New York, NY, USA) was used for quantification co-localized proteins. All the stained cells were from multiple biological replicates, each consisting of cells from at least three different donors per pooled experiment and randomly selected from multiple technical replicates for imaging.

Loss of function by siRNA oligonucleotides

CD4+ T cells were nucleofected at the day of cell isolation with non-targeting siScramble (5′-GGCGGCTTTGTAGGATTCG-3′, Sigma-Aldrich) or pool of two siGIMAP4 gene-specific (GIMAP4HSS124340 and GIMAP4HSS124341, Thermo Fisher, Invitrogen) RNAi oligos (1.5 ng/4×10⁵ cells/transfection). All nucleofections were at a cell density of 4×10⁵/100 μl Opti-MEM 1 (Thermo Fisher, Invitrogen) using the Nucleofector Device (program U-14, Amaxa, Lonza, Colonge, Germany). The cells (1.5–2×10⁶ cell ml⁻¹) were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U ml⁻¹ penicillin/100 μg ml⁻¹ streptomycin (#P0781, Sigma-Aldrich) and 2 mM L-glutamine prior to activation at 37°C for 43 h. The cells were harvested, activated, induced to differentiate and cultured as described above.

Transcriptomic profiling and analysis

Samples from three independent biological cultures were harvested at 43 h after nucleofection and at 2 h from CD3/anti-CD28 activation and IL-12 addition. GIMAP4 downregulation was verified by western blotting at 43 h after
nucleofection. The sample treatments were performed at the Finnish Microarray and Sequencing Centre, Turku, Finland. Total RNA was isolated with the Rneasy mini kit (Qiagen, Valencia, CA, USA), and sample preparation for GeneChip oligonucleotide array hybridization was performed according to Affymetrix’s instructions using 250 ng of total RNA as a starting material with the GeneChip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA, USA). Samples were hybridized to HG-U219 arrays using a GeneTitan Instrument (Affymetrix). Raw data were normalized using robust multi-array average algorithm as implemented in Bioconductor suite. Duplicate and un-annotated probe sets were removed using the genefilter package in R. In case of duplicates, the probe set with the highest inter quartile range was retained. Present and absent calls for probe sets were generated by fitting the chip-wide expression data to a two-component Gaussian distribution function using the standard EM algorithm implemented in mixtools package in R. A probe set was defined to be present if it has an expression value higher than the threshold where the two components of the Gaussian distribution densities meet. Differential expression analysis was done using the unpaired, moderated t-test as implemented in limma with R. A gene was considered to be differentially expressed if Benjamini–Hochberg adjusted P-value is <0.05 and log fold change is <−1 or >1. The GIMAP4 transcriptomic data can be accessed at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vilheenwuiqota&acc=GSE45720.

Extracellular protein profiling

Prior to cytokine profiling, the efficacy of GIMAP4 knockdown was verified by western blotting at 24 h after anti-CD3/anti-CD28 activation. An aliquot of cell culture medium (200–400 μl) was collected daily up until 5th day of culture, stored at −70°C and the concentrations of secreted cytokines were analysed with MILLIPLEX MAP-Kit (MPXHCYT0-60K, Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Tumour necrosis factor-β, IL-13 and IL-10 were measured from one biological replicate and IFN-γ from three biological replicates. The detection of cytokines by Luminex 100 IS 2.2 (Luminex, Austin, TX, USA) was performed in Plexalaboratory, Medicity, University of Turku. The significance of the IFN-γ secretion differences were calculated by using the two-tailed t-test.

Western blotting

Samples were lysed with in Triton-X-100 lysis buffer (50 mmv Tris–HCl, pH 7.5, 150 msv NaCl, 0.5% Triton-X-100, 5% glycerol, 1% sodium dodecyl sulphate, 1 msv Na3VO4, 10 msv NaF) supplemented by 1 × Complete Mini protease inhibitor (Roche no. 11-836-170-001, Roche, Basel, Switzerland) and 1 × PhosphoStop (Roche no. 04-906-837-001) and incubated at +97 °C for 7 min and sonicated with 0.5-min pulses for 5 min. Protein samples were loaded equally to 12% sodium dodecyl sulphate gels in 1 × sodium dodecyl sulphate sample buffer. Separated proteins were transferred to a nitrocellulose membrane (Hybond enhanced chemiluminescence; GE Healthcare) and detected by using 1:5000 dilution of rabbit IgG (Cell Signaling Technology Inc., Danvers, MA, USA) and anti-β 2 and CXCR3 were measured by FACSCalibur Flow Cytometer figures; MTH, RL and TAH interpreted data; and TAH and RL contributed reagents.

Flow cytometric analyses

The FITC BrdU Flow kit (BD Pharmingen, San Jose, CA, USA) was used according to the manufacturer’s instructions to detect the cell viability. For iGimap4-treated and siScramble-treated cells to sample, cultured for 48 and 72 h, BrdU was added at the point of anti-CD3/anti-CD28 activation and IL-12 addition. Active caspase-3 was measured according to the manufacturer’s instructions (catalog number 550914, BD Pharmingen) from non-stimulated siGimap4 and non-targeting siScramble controls before initial anti-CD3/anti-CD28 activation and from unstimulated, unstimulated and IL-12-supplemented, Brefeldin A-stimulated and Brefeldin A-stimulated IL-12-supplemented siGimap4- and siScramble-treated cells. The intracellular expression of IFN-γ was measured from siGimap4- and siScramble-treated cells, with supplemented IL-12. The expression was measured at 48 and 72 h after initial anti-CD3/anti-CD28 activation. Five hours after adding Brefeldin A, the cells were incubated with FITC-conjugated mouse α-human-IFN-γ (BD Pharmingen no. IFG601) or isotype control (BD Pharmingen no. MG010). The surface expression of IL12R2 was measured from siGimap4- and siScramble-treated cells, with supplemented IL-12 and with no supplements, at 24, 48 and 72 h after initial anti-CD3/anti-CD28 activation by first incubating with rat α-human CXCXR3-APC (BD Pharmingen) or isotype control mouse IgG1k-APC (BD Pharmingen). In all, 0.1–0.5 × 10⁶ cells were used for each flow cytometry staining, and at least 10,000 cell counts were measured per sample. The incorporated BrdU and the expression of active caspase 3, IFN-γ, IL12R2 and CXCR3 were measured by FACSCalibur Flow Cytometer and analysed with the CellQuest Pro (BD Pharmingen). The significance of the difference was calculated from three biological replicates by using a two-tailed t-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: MTH, TAH and RL designed the research and wrote the paper; MTH and TAH performed experiments; KK and HFL were responsible for transcriptionics data analysis and contributed to writing; MTH made figures; MTH, RL and TAH interpreted data; and TAH and RL contributed reagents.
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