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ER protein translocation and retrotranslocation are crucial to inflammatory effector CD4⁺ T cell function.

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Abstract

Effector CD4⁺ T cells underpin the pathogenesis of autoimmune diseases such as multiple sclerosis. These cells can be classified by the cytokines they secrete, with T helper 1 (Th1) cells generating interferon (IFN) γ and Th17 cells secreting interleukin (IL)-17. The endoplasmic reticulum (ER) has been implicated as a potentially crucial site in regulating CD4⁺ T cell function. Secretory and transmembrane proteins are shuttled into the ER via the Sec61 translocon, where they undergo appropriate folding; misfolded proteins are dislocated from the ER in a p97-dependent manner. Here we provide evidence that both processes are crucial to the secretion of inflammatory cytokines from effector CD4⁺ T cells. The pan-ER inhibitor eeeyarestatin-1 (ESI), which interferes with both Sec61 translocation and p97 dislocation, inhibited secretion of interferon (IFN) γ , interleukin (IL)-2 and tumor necrosis factor (TNF) α from Th1 cells in a dose-dependent manner. Selective inhibition of Sec61 by apratoxin A (ApraA) revealed that ER translocation is crucial for Th1 cytokine secretion, and that inhibition of p97 by NMS-873 also inhibited Th1 function, albeit to a lesser degree. By contrast, none of ESI, ApraA or NMS-873 could significantly reduce IL-17 secretion from Th17 cells. Both ApraA and NMS-873 reduced phosphorylation of Stat1 in Th1 cells, indicating the involvement of both translocation and dislocation in Th1 differentiation pathways. ApraA, but not NMS-873, diminished phosphorylation of the Th17 transcription factor Stat3. Surprisingly, despite its modest effects on Th1/Th17 function in vitro, NMS-873 was able to reduce disease severity in CD4⁺ T cell-driven experimental autoimmune encephalomyelitis (EAE). Together, our data indicate that CD4⁺ T cell function, and Th1 cell function in particular, is dependent on protein translocation and dislocation across the ER.

Introduction

CD4⁺ T cells play essential roles in directing the mammalian immune response to infection, yet they also underpin the pathobiology of autoimmune disorders such as multiple sclerosis (MS) and type I diabetes. Upon activation by cognate antigen presented on MHC class II, CD4⁺ T cells differentiate into effector subsets that are distinguishable by their cytokine secretion profile. Interferon (IFN) γ -secreting Th1 and interleukin (IL)-17-secreting Th17 cells are the subsets most relevant to autoimmune disease[1]. Studying the molecular regulation of these cells is essential to obtaining a detailed understanding on how autoimmune pathologies unfold.

The endoplasmic reticulum (ER) is a major proteostatic hub in the cell, which is responsible for the appropriate folding of integral membrane and secreted proteins[2], and thus plays a key role in the generation of secreted cytokines. Nascent polypeptides containing an ER-targeting sequence, the signal peptide, are transported (*translocated*) into the ER lumen via the action of the heterotrimeric Sec61 ER membrane channel[3]. While properly folded proteins are targeted to Golgi bodies for further sorting, misfolded proteins are dislocated (*retro-translocated*) back into the cytoplasm by the AAA⁺ ATPase p97 as the last step of the ER-associated degradation (ERAD) pathway [4]. ERAD is a key quality-control mechanism that prevents the accumulation and aggregation of misfolded proteins and subsequent ER stress [5]. Both translocation and retrotranslocation have been implicated in T cell function: expression of IFN γ by T cells is Sec61-dependent[6], while the T cell receptor (TcR) α -chain is a substrate of VCP-mediated dislocation[4].

In this work, using inhibitors of Sec61 translocation, retro-translocation, or both, we assessed how these processes can regulate CD4⁺ T cell function. We found that translocation and to a lesser extent, retro-translocation, were critical for inflammatory cytokine secretion production

in Th1 cells *in vitro*. By contrast, ER transport was less important for Th17 function. Notably, *in vivo* inhibition of retro-translocation reduced disease severity in a CD4⁺ T cell mediated animal model of MS. Together, our data indicate that translocation and retro-translocation across the ER are targetable processes that can promote CD4⁺ effector T cell fates.

Results

Translocation and/or retrotranslocation across the ER augment effector CD4⁺ T cell function

To determine whether ER import and/or quality control could impact effector T cell function, we first assessed the *in vitro* effects of the small molecule inhibitor eeyarestatin I (ESI) [7], which targets Sec61 [8,9] as well as p97 [10,11] and thus can inhibit both translocation and retro-translocation. We generated effector CD4⁺ T cell subsets from naïve CD4⁺CD62L^{hi} precursors by stimulating them with agonistic antibodies to CD3 and CD28 under defined Th1 or Th17 differentiation conditions for 5 days [12]. This approach eliminated the need for feeder antigen-presenting cells (APCs); as translocation can modulate APC function [13], this permitted us to isolate the effects of the inhibitor on T cells alone.

Acute (4 hour) stimulation of Th1 cells with ESI strongly perturbed their capacity to generate the signature cytokine IFN γ as measured by flow cytometry. The percentage of IFN γ -positive cells was substantially reduced, relative to controls, at 2 μ M, the lowest concentration studied. At higher concentrations, IFN γ production was completely ablated (Figure 1A). At all concentrations studied, ESI sharply diminished the frequency of Th1 cells that were positive for

the autocrine T cell growth factor IL-2 (Figure 1B), and ESI additionally reduced the percentage of cells positive for the inflammatory effector cytokine TNF α (Figure 1C).

We next wanted to examine the effects of ESI on inflammatory cytokine generation from Th17 cells. We therefore differentiated CD4⁺CD62L^{hi} T cells under Th17 conditions and again exposed them acutely to ESI. As it can be challenging to detect IL-17 by flow cytometry from Th17 cells when they are generated *in vitro* under APC-free conditions, we measured its secretion into culture supernatant by ELISA. Interestingly, production of this signature Th17 cytokine was unaffected by ESI (Figure 2A). Higher doses of ESI did reduce the frequency of TNF α ⁺ Th17 cells (Figure 2B), but to a lesser degree than that seen in Th1 cells (Figure 1C). Altogether, our data indicates that concurrent inhibition of both translocation and retro-translocation disrupted the inflammatory function of Th1 cells and, to a lesser extent, Th17 cells.

Translocation is essential for Th1 cytokines but dispensable for IL-17 production

Our data thus far showed that the multifunctional ER protein transport inhibitor ESI could repress inflammatory cytokine expression from effector CD4⁺ T cells. We next sought to separately interrogate the contributions of translocation versus retrotranslocation to T cell function. The cyanobacteria-derived compound apratoxin A (ApraA) blocks insertion of polypeptides into the ER by specifically targeting the luminal end of Sec61 α [14] and thus permits one to dissect the role of translocation to cellular processes. We thus examined whether ApraA could repress inflammatory cytokine production from Th1 cells. Indeed, the frequency of IFN γ ⁺ (Fig. 3A), IL-2⁺ (Fig 3B) and TNF α ⁺ Th1 cells was reduced upon ApraA treatment at all concentrations studied. This was in agreement with previous observations that IFN γ [6] and TNF α [15] are clients for

Sec61-mediated insertion into the ER and that the Sec61 inhibitor mycolactone could ablate IFN γ production from T cells [6]. Intriguingly, while longer-term treatment with mycolactone was required to diminish IL-2 generation from T cells[16], we observed a near complete shutdown of IL-2 after acute stimulation with ApraA.

We next examined the effect of ApraA treatment on Th17 cells. Similar to ESI, we found that ApraA did not significantly alter IL-17 secretion (Figure 4A). Intriguingly, TNF α production from ApraA-treated Th17 cells was completely ablated (Figure 4B). While this was in keeping with the observation that TNF α is inserted into the ER in a Sec61-dependent manner [15], it was notable that in Th1 cells, a residual frequency of TNF α -producing cells were seen upon ApraA treatment.

Retro-translocation promotes Th1 cytokine production

Thus far, we had shown that the phenotype of ESI- and ApraA-treated cells overlapped substantially, albeit not perfectly. However, as ESI additionally blocks p97-dependent extraction of misfolded substrates from the ER, these findings did not rule out a downstream role for retrotranslocation in regulating the production of inflammatory cytokines from effector CD4⁺ T cells. We therefore next examined the effects of NMS-873, a potent and specific inhibitor of p97, on Th1 and Th17 cytokine production. Acute inhibition of p97 reduced the frequency of IFN γ -positive Th1 cells (Figure 5A); however, this suppression was not as stark as that induced by ApraA. IL-2 was completely inhibited by NMS-873 (Figure 5B), as was the case for both ESI and ApraA. The percentage of TNF α ⁺ Th1 cells was reduced in a dose-dependent manner (Figure 5C), but the effect was far more modest than that seen in Apra-treated cells. As was the case for ApraA,

IL-17 production from Th17 cells was refractory to inhibition by NMS-873 (Figure 6A). However, again the effect of NMS-873 on TNF α production was modest (Figure 6B). Together, our data indicated that retrotranslocation of misfolded substrates can augment the production of inflammatory cytokines from Th17 cells, but that the contribution of this process is likely less than that of translocation.

Translocation and retrotranslocation trigger Stat1 activation.

Effector CD4⁺ T cell differentiation is dependent on the downstream activation of transcription factors of the Stat family, with phosphorylation of Stat1 being crucial for Th1 responses. Our data thus far indicated that both translocation and retrotranslocation were important for the generation of the signature Th1 cytokine IFN γ ; however, multiple lines of evidence indicate that Th1 cells can retain inflammatory function in the absence of IFN γ [17] and IFN γ -IFN γ R autocrine signaling [18]. Activation of the transcription factor Stat1 is a crucial step in Th1 differentiation. Thus, we wanted to examine whether ER protein insertion and/or dislocation are essential to this checkpoint. We found that acute treatment with ApraA abrogated the phosphorylation of pStat1 in primary Th1 cells (Figure 7A). This was in agreement with previous findings that mycolactone could downregulate pStat1 in Jurkat T cells [6]. Intriguingly, NMS-873 also reduced pStat1 levels (Figure 7B), indicating that retrotranslocation was also required for optimal differentiation of Th1 cells.

Translocation, but not retro-translocation, is required for optimal Th17 differentiation

We next wanted to assess whether translocation and retro-translocation were required for Th17 differentiation. We thus assessed activation (phosphorylation) of Stat3, the critical transcription factor for Th17 responses. Notably, despite having no significant effect on IL-17 expression from Th17 cells, ApraA reduced their levels of pStat3 (Figure 8A). NMS-873, by contrast, had no effect on pStat3 (Figure 8B). Together, our data demonstrated that while both translocation and retro-translocation were important for Th1 differentiation, translocation alone was needed for optimal Th17 differentiation.

NMS-873 reduces the severity of experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a mouse model of the immune aspects of multiple sclerosis pathogenesis that is induced by immunization of susceptible mouse strains with myelin-derived autoantigen. An autoimmune disease, it is also a well-established model with which to study inflammatory CD4⁺ T cell function as it implicates both Th1 and Th17 effector mechanisms [19]. We therefore used the EAE model to study the role of ER protein transport in regulating inflammatory T cell function *in vivo*. ApraA was previously shown to be toxic in mice [20], precluding our ability to study its effects. We therefore examined the ability of NMS-873 to modulate the severity of EAE in C57BL6/J mice that were immunized with the encephalitogenic peptide MOG_[35-55]. A single treatment with NMS-873 prior to the onset of symptoms was sufficient to reduce disease severity in EAE mice, relative to vehicle-treated controls (Figure 9). These data show that retrotranslocation augments CD4⁺ T cell-driven autoimmunity, though the effects of translocation remain to be elucidated.

Discussion

Identifying regulatory pathways important to Th1 and/or Th17 cell function could pave the way for small-molecule therapies for T cell-driven autoimmune diseases. As cytokine production is essential to their activity, and as the ER is critical to the folding and quality control of secreted proteins, it is logical to consider that translocation and retro-translocation of polypeptides across the ER lumen may be critical to effector T cell function. Here, we show that ESI can potently suppress the production of IFN γ , IL-2 and TNF α from Th1 cells. ESI is of potential therapeutic interest because of its ability to control cancer cell growth, both *in vitro* and in preclinical models [21-23]. However, it has been reported as repressing both Sec61 [8,9] and p97 [10,11], making it difficult to assess whether translocation, retro-translocation, or both, are responsible for its effects.

To address each mechanism separately, we first considered the effects of the Sec61-specific inhibitor ApraA on Th1 cell function and found that it phenocopied ESI at concentrations that were 10-fold lower. These findings are in line with the previous observation that the macrolide mycolactone, which is a Sec61 inhibitor reduces production of the Th1 signature cytokine IFN γ from T cells *in vitro* and *in vivo* [6], and downregulates generation of TNF α , albeit from macrophages [24]. The effect of mycolactone is Sec61-specific, as expression of a mutant protein that lacks the putative mycolactone binding site rendered T cells refractory to the IFN γ -inhibitory effects of the drug [6]. Our observations extend these findings in two important ways. First, mycolactone was shown to inhibit mycolactone on IFN γ in parallel with the inhibition of CD62L [6], a naïve and central memory T cell surface marker that is not expressed by effector T cells [25]. This left open the question of whether Sec61-dependent translocation is essential to the production

of IFN γ in effector T cells. Here, using a well-established differentiation protocol [12], we demonstrate that Sec61 is indeed essential for IFN γ generation by effector Th1 cells. Further, while mycolactone can inhibit IL-2 production upon stimulation with anti-CD3 and anti-CD28, it only does so after prolonged exposure [16], suggesting that its effects on this cytokine are indirect. By contrast, we find that relatively short (4 hour) treatment with ApraA can completely ablate IL-2 generation from Th1 cells.

Importantly, we also observed a role for retro-translocation of misfolded proteins in mediating inflammatory cytokine production from Th1 cells, as NMS-873 suppressed IFN γ , IL-2 and TNF α and additionally downregulated the activation of Stat1. Retro-translocation is an essential cellular quality control mechanism as it prevents the accumulation of misfolded proteins in the ER. Such accumulation leads to ER stress, which in turn leads to a shutdown of protein translation and the induction of the unfolded protein response (UPR), a signaling cascade characterized by the activation of effector proteins such as PERK, ATF6 or IRE1 α [26]. Our data would therefore indicate that p97 promotes Th1 responses while the UPR represses them. While selective ablation/inhibition of PERK or IRE1 α does not inhibit IFN γ , broad induction of the cellular stress response, by glucose deprivation or glycolysis inhibition, does perturb Th1 cell responses. This would suggest that blockade of p97-dependent ERAD might trigger global upregulation of UPR players, subsequently leading to a suppression of Th1 effector function. Crucially, we found that NMS-873 ameliorates symptom severity on the EAE model of MS. This is therefore, to our knowledge, the first report that inhibition of p97 can reduce the severity of T cell-driven autoimmune disease.

It is notable that none of the inhibitors studied could suppress the production of IL-17 from Th17 cells. However, ApraA inhibited the activation of Stat3 in these cells, suggesting that

translocation can indeed impact Th17 differentiation. While IL-17 is considered the hallmark Th17 cytokine, its expression is not essential for Th17 function; in fact, in the *in vivo* setting, highly pathogenic Th17 cells may lose IL-17 expression entirely and instead transition to an “ex-Th17” phenotype in which they produce IFN γ [27-29]. The *in vivo* toxicity of ApraA [20] precludes our ability to study its effects on T cell function in autoimmunity. In the future it may be interesting to assess the role of ApraA, and NMS, in T cell adoptive transfer models of EAE in which myelin antigen-specific T cells are restimulated *ex vivo* prior to their introduction to immunologically naïve recipients.

In conclusion, our data demonstrate a crucial role for polypeptide translocation and retrotranslocation in the function of effector T cells, and for Th1 cells in particular. The targeting of Sec61- and p97-dependent pathways may provide promising avenues for the treatment of inflammatory T cell-dependent diseases.

Acknowledgments

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Conflicts of interest

242 The authors declare no conflict of interest.

243

244 **Materials and methods**

245

246 *Animals and ethics*

247 All experimental protocols and breedings were approved by the Animal Protection Committee of
248 the Centre de recherche du CHU de Québec - Université Laval (protocols 2017-037-2 and 2017-
249 090-2). 6–8 week old B6 (C57BL/6) mice were obtained from Charles River.

250

251 *Reagents*

252 Flow cytometry monoclonal Abs (mAbs) against mouse antigens were obtained from eBioscience
253 (CD4, clone RM4-5; CD62L; clone MEL-14; IFN γ , clone XMG1.2; TNF α , clone MP6-XT22), or
254 BD (IL-2, clone JES6-5H4). The following recombinant cytokines were used for *in vitro* T cell
255 cultures: recombinant mouse (rm) IL-12 (R&D Biosystems), rmIL-2 (Miltenyi), recombinant
256 human (rh) TGF β (Miltenyi), rmIL-6 (Peprotech), rmIL-23 (R&D Biosystems). The following
257 mAbs, all obtained from BioXcell, were used for *in vitro* T cell cultures: anti-CD3 (clone 145-
258 2C11), anti-CD28 (clone 37.51), anti-IL-4 (clone 11B11) and anti-IFN γ (XMG1.2). NMS873 (Cat.
259 No. 6180) and ErsI (Cat. No. 3922) were purchased from Tocris. Apratoxin A was prepared as
260 previously described [14].

261

262 *T cell isolation and differentiation*

263 CD4⁺CD62L^{hi} T cells were enriched from spleen and lymph nodes of B6 mice using Naïve CD4⁺
264 T cell Isolation kit (Miltenyi). Cells were then cultured in DMEM (Life Technologies) plus 10%
265 fetal calf serum (Corning), supplemented as previously described [30], and stimulated for 2 days
266 with tissue culture plate-bound 2 µg mL⁻¹ anti-CD3 and anti-CD28 (BioXcell) under either Th1
267 (10 ng mL⁻¹ rmIL-12 + 10 µg mL⁻¹ anti-IL-4) or Th17 (3 ng mL⁻¹ rhTGFβ + 20 ng mL⁻¹ rmIL-6 +
268 20 µg mL⁻¹ anti-IFNγ) conditions [31]. After 2 days, cells were transferred to non-coated plates in
269 the presence of 10 ng mL⁻¹ rmIL-2 (for Th1) or 20 ng mL⁻¹ rmIL-23 (for Th17) for an additional
270 3 days. At d5 cells, were treated with ESI, ApraA or NMS873 for 4 hours. Equivalent volume of
271 DMSO was used as a vehicle control in all cases. Cells were subsequently assessed by flow
272 cytometry, ELISA or Western blot.

273

274 *Cytokine analysis*

275 For detection of IFNγ, IL-2 or TNFα, cells were treated with 50 ng mL⁻¹ phorbol 12-myristate 13-
276 acetate (Sigma-Aldrich), 1 µM ionomycin (Sigma-Aldrich) and GolgiStop (1 µL per mL culture;
277 BD Biosciences) for 4 hrs [32]. After 4 hrs, cells were subsequently incubated with Fc Block
278 (Biolegend) and Fixable Viability Dye (eBioscience). Cells were stained for surface CD4 were
279 then fixed and permeabilized using Fixation and Perm/Wash buffers (eBioscience). They were
280 then stained with fluorescent Abs against IFNγ, IL-2 or TNFα. All FACS acquisition was
281 performed on a LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo
282 software (Tree Star). All FACS data presented are gated on live CD4⁺ events. For detection of IL-
283 17, ELISA-MAX Deluxe IL-17A detection kit (Biolegend) was used.

284

285

286

287 *Western blotting*

288 Whole cell extracts were prepared using cell lysis buffer (RIPA buffer, Boston BioProducts; 10%
289 SDS; 100mM Na₃VO₄; 100 mM PMSF; 1X Halt Protease/Phosphatase Inhibitor Cocktail,
290 ThermoScientific). Lysates were boiled, clarified and quantitated using DC Protein Assay (Bio-
291 Rad). Equivalent amounts of protein were loaded onto Criterion TGX precast gels (Bio-Rad),
292 which were subsequently transferred to PVDF (Bio-Rad). After incubation with 5% nonfat milk
293 in TBS plus 0.5% Tween for 60 min, the membrane was washed once with TBST and incubated
294 with antibodies against Stat1(1:1000), pStat1 (1:1000), Stat3 (1:1000) or pStat3(1:2000) at 4 °C
295 overnight. Membranes were washed three times for 10 min and incubated with a 1:2000 dilution
296 of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 1 h. Blots were
297 washed with TBST three times and developed with the ECL system (Millipore) according to the
298 manufacturer's protocols. Chemiluminescence was detected using a MyECL image analyzer
299 (ThermoFisher).

300

301 *EAE induction*

302 EAE was induced by active immunization in B6 mice at 8-12 weeks of age as described previously
303 [33]. Briefly, mice were immunized with 200 ug MOG_[35-55] (CHU de Québec Medicinal
304 Chemistry Platform) in 100 µL of complete Freund's adjuvant (Difco) supplemented with 500 µg
305 M. tuberculosis extract (Difco). Mice received 200 ng pertussis toxin (List Biological Labs) on d0
306 and d2. Immunized mice received either 0.2mg kg⁻¹ NMS873 or equivalent volume of DMSO, i.v.,

on d5 post-immunization. Mice were observed daily for signs of EAE. Mice were weighed and assessed for disease symptoms daily for up to 20 days, using an established semi-quantitative scale: 0, no disease; 1, decreased tail tone; 2, hind limb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, front and hind limb paralysis; 5, moribund or dead [32].

Statistical Analysis

Flow cytometric and ELISA data were analyzed by two-way ANOVA followed by Sidak's multiple corrections test. Error bars represent standard error (s.e.m.). Differences in EAE disease curve were analyzed by linear regression. All statistical analyses were performed using Prism (GraphPad).

Figure legends

Figure 1. Effects of ESI on Th1 cells. CD4⁺CD62L^{hi} T cells were stimulated in vitro for 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions. Cells were then treated for 4 hours with the indicated dose of ESI, or the equivalent volume of DMSO vehicle, and were assessed for the production of IFN γ (**A**), IL-2 (**B**) or TNF α (**C**) by intracellular flow cytometry, gated on live CD4⁺ events. Representative of 3 experiments. Bar graphs represent triplicate, individual, culture wells from a representative experiment. ****, $p < 0.0001$, Sidak's multiple comparisons test after 2-way ANOVA. Error bars represent s.e.m.

Figure 2. Effects of ESI on Th17 cells. CD4⁺CD62L^{hi} T cells were stimulated in vitro for 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions. Cells were then treated for 4 hours with the indicated dose of ESI, or the equivalent volume of DMSO vehicle. **A.** IL-17 secretion into culture supernatant was assessed by ELISA. **B.** TNFα was assessed by intracellular flow cytometry, gated on live CD4⁺ events. Representative of 3 experiments. Bar graphs represent triplicate, individual, culture wells from a representative experiment. ****, p<0.0001, Sidak's multiple comparisons test after 2-way ANOVA. Error bars represent s.e.m.

Figure 3. Effects of Sec61 inhibitor ApraA on Th1 cells. CD4⁺CD62L^{hi} T cells were stimulated in vitro for 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions. Cells were then treated for 4 hours with the indicated dose of ApraA, or the equivalent volume of DMSO vehicle, and were assessed for the production of IFNγ (**A**), IL-2 (**B**) or TNFα (**C**) by intracellular flow cytometry, gated on live CD4⁺ events. Representative of 3 experiments. Bar graphs represent triplicate, individual, culture wells from a representative experiment. ****, p<0.0001, Sidak's multiple comparisons test after 2-way ANOVA. Error bars represent s.e.m.

Figure 4. Effects of ApraA on Th17 cells. CD4⁺CD62L^{hi} T cells were stimulated in vitro for 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions. Cells were then treated for 4 hours with the indicated dose of ApraA, or the equivalent volume of DMSO vehicle. **A.** IL-17 secretion into culture supernatant was assessed by ELISA. **B.** TNFα was assessed by intracellular flow cytometry, gated on live CD4⁺ events. Representative of 3 experiments. Bar

348 graphs represent triplicate, individual, culture wells from a representative experiment. ****,
349 $p < 0.0001$, Sidak's multiple comparisons test after 2-way ANOVA. Error bars represent s.e.m.

350

351 **Figure 5. Effects of p97 inhibitor NMS-873 on Th1 cells.** CD4⁺CD62L^{hi} T cells were stimulated
352 *in vitro* for 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions. Cells were
353 then treated for 4 hours with the indicated dose of NMS-873, or the equivalent volume of DMSO
354 vehicle, and were assessed for the production of IFN γ (A), IL-2 (B) or TNF α (C) by intracellular
355 flow cytometry, gated on live CD4⁺ events. Representative of 3 experiments. Bar graphs represent
356 triplicate, individual, culture wells from a representative experiment. *, $p < 0.05$, **, $p < 0.01$, ***,
357 $p < 0.001$, ****, $p < 0.0001$, Sidak's multiple comparisons test after 2-way ANOVA. Error bars
358 represent s.e.m.

359

360 **Figure 6. Effects of NMS-873 on Th17 cells.** CD4⁺CD62L^{hi} T cells were stimulated *in vitro* for
361 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions. Cells were then treated
362 for 4 hours with the indicated dose of NMS-873, or the equivalent volume of DMSO vehicle. A.
363 IL-17 secretion into culture supernatant was assessed by ELISA. B. TNF α was assessed by
364 intracellular flow cytometry, gated on live CD4⁺ events. Representative of 3 experiments. Bar
365 graphs represent triplicate, individual, culture wells from a representative experiment. ****,
366 $p < 0.0001$, Sidak's multiple comparisons test after 2-way ANOVA. Error bars represent s.e.m.

367

368 **Figure 7. Effects of ApraA and NMS-873 on Stat1 activation.** CD4⁺CD62L^{hi} T cells were
369 stimulated *in vitro* for 5 days with anti-CD3 and anti-CD28 under Th1 differentiation conditions.

370 Cells were then treated for 4 hours with either ApraA (**A**) or NMS-873 (**B**) and assessed by
 371 Western blot for presence of phospho-Stat1 (pStat1) or total Stat1. Indicated marker sizes are in
 372 kDa. Stat1 is 91 kDa. **A.** *Lane 1*, 0.2 μ M ApraA; *lane 2*; 0.4 μ M ApraA; *lane 3*, 0.6 μ M ApraA;
 373 *lane 4*, DMSO control for lane 1; *lane 5*, DMSO control for lane 2; *lane 6*, DMSO control for lane
 374 3. **B.** *Lane 1*, 2 μ M NMS-873; *lane 2*; 4 μ M NMS-873; *lane 3*, 6 μ M NMS-873; *lane 4*, DMSO
 375 control for lane 1; *lane 5*, DMSO control for lane 2; *lane 6*, DMSO control for lane 3.

376

377 **Figure 8. Effects of ApraA and NMS-873 on Stat3 activation.** CD4⁺CD62L^{hi} T cells were
 378 stimulated *in vitro* for 5 days with anti-CD3 and anti-CD28 under Th17 differentiation conditions.
 379 Cells were then treated for 4 hours with either ApraA (**A**) or NMS-873 (**B**) and assessed by
 380 Western blot for presence of phospho-Stat3 (pStat3) or total Stat3. Indicated marker sizes are in
 381 kDa. Stat3 is 86 kDa. **A.** *Lane 1*, 0.2 μ M ApraA; *lane 2*; 0.4 μ M ApraA; *lane 3*, 0.6 μ M ApraA;
 382 *lane 4*, lane 4, 0.8 μ M ApraA; *lane 5*, DMSO control for lane 1; *lane 6*, DMSO control for lane 2;
 383 *lane 7*, DMSO control for lane 3; *lane 8*, DMSO control for lane 4. **B.** *Lane 1*, 2 μ M NMS-873;
 384 *lane 2*; 4 μ M NMS-873; *lane 3*, 6 μ M NMS-873; *lane 4*, lane 4, 8 μ M NMS-873; *lane 5*, DMSO
 385 control for lane 1; *lane 6*, DMSO control for lane 2; *lane 7*, DMSO control for lane 3; *lane 8*,
 386 DMSO control for lane 4.

387

388 **Figure 9. NMS-873 treatment reduces the severity of EAE.** C57BL6/J (B6) mice were
 389 immunized with 200 μ g MOG[35-55] peptide in complete Freund's adjuvant and were boosted
 390 with 200 ng pertussis toxin at d0 and d2. At d5 post-immunization, mice received either NMS-873
 391 (0.2 mg kg⁻¹; n=5) or an equivalent volume DMSO (n=5). Mice were monitored daily for signs of

EAE. Top graph, mean daily scores. Error bars represent s.e.m. Bottom graph, linear regression analysis of the disease curves. **** $p < 0.0001$.

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Figure 1

Figure 2

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Figure 3

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Figure 5

Figure 6

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Figure 7

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Figure 8

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Figure 9