Statin-induced Kruppel-like factor 2 expression in human and mouse T cells reduces inflammatory and pathogenic responses

De-xiu Bu,[1] Margarite Tarrio,[1] Nir Grabie,[1] Yuzhi Zhang,[1] Hiroyuki Yamazaki,[2] George Stavrakis,[1] Elena Maganto-Garcia,[1] Zachary Pepper-Cunningham,[1] Petr Jarolim,[1] Masanori Aikawa,[2] Guillermo García-Cardeña,[1] and Andrew H. Lichtman[1]

[1]Department of Pathology and [2]Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts, USA.

The transcription factor kruppel-like factor 2 (KLF2) is required for the quiescent and migratory properties of naive T cells. Statins, a class of HMG-CoA reductase inhibitors, display pleiotropic immunomodulatory effects that are independent of their lipid-lowering capacity and may be beneficial as therapeutic agents for T cell–mediated inflammatory diseases. Statins upregulate KLF2 expression in endothelial cells, and this activity is associated with an antiinflammatory phenotype. We therefore hypothesized that the immunomodulatory effects of statins are due, in part, to their direct effects on T cell *KLF2* gene expression. Here we report that lipophilic statin treatment of mouse and human T cells increased expression of KLF2 through a HMG-CoA/prenylation–dependent pathway. Statins also diminished T cell proliferation and IFN-γ expression. shRNA blockade of *KLF2* expression in human T cells increased IFN-γ expression and prevented statin-induced IFN-γ reduction. In a mouse model of myocarditis induced by heart antigen–specific CD8$^+$ T cells, both statin treatment of the T cells and retrovirally mediated overexpression of KLF2 in the T cells had similar ameliorating effects on disease induction. We conclude that statins reduce inflammatory functions and pathogenic activity of T cells through KLF2-dependent mechanisms, and this pathway may be a potential therapeutic target for cardiovascular diseases.

Introduction

Kruppel-like factor 2 (KLF2) is a member of a transcription factor family, with homology to the drosophila kruppel transcription factor. It is expressed in lung, endothelial cells, and lymphocytes and is essential for blood vessel integrity and lung development (1). *Klf2*-null mice exhibit abnormal blood vessel formation, resulting in embryonic hemorrhage and death (1). In addition to its role in lung and vascular development, KLF2 appears to play several roles in the regulation of T cell function. One of the first of these roles to be described is the inhibition of proliferation. Ectopic expression of the *KLF2* gene in the constitutively proliferative human T cell leukemia line Jurkat decreases mitotic activity of these cells (2, 3). Furthermore, gene-targeted KLF2-deficient mouse T cells have a hyper proliferative phenotype (2, 3). Several lines of evidence indicate that KLF2 is required for the maintenance of T cell quiescence. *KLF2* mRNA is expressed in naive and memory T cells and is rapidly downregulated upon TCR stimulation of these cells (4, 5). Although most of the functions ascribed to KLF2 indicate that KLF2 is required to maintain the nonactivated phenotype, some data suggest a more complicated set of functions. For example, KLF2 may also play a role in promoting the very early stages of T cell activation, at which time its expression is transiently increased in Jurkat cells, and it transactivates IL-2 promoter activity (6). Furthermore, the transition from effector to memory stages of T cell responses may involve KLF2 expression in effector cells before the memory phenotype is established, as described in mouse CD8$^+$ T cells (5).

Due to the embryonic lethality of global KLF2 deficiency, the function of KLF2 in T cells has been studied in mice with selective deficiency of KLF2 only in hematopoietic cells (7) or only in lymphocytes (8–10). In all these cases, there is relatively normal T cell development in the thymus but a severe T cell deficiency in the periphery. This deficiency has been attributed to defective expression of sphingosine-1-phosphate (S1P) receptor 1 (S1PR1), which is required for S1P-mediated egress of T cells from the thymus and peripheral lymphoid organs. Other T cell homing defects in these mice have also been attributed to a lack of KLF2-dependent CD62L expression, which is required for naive T cell migration into lymph nodes. Other abnormalities in KLF2-deficient T cell expression that have been reported in individual studies, such as enhanced Fas ligand–mediated apoptosis (8) and expression of inflammatory chemokine receptors, leading to constitutive T cell migration into nonlymphoid tissues (9), have not been consistently seen in other studies (10). Overall, work performed with KLF2-deficient T cells in vivo indicates the importance of KLF2 expression for normal peripheral T cell recirculation but does not clarify how KLF2 modulates mature peripheral T cell function.

Statins, a class of HMG-CoA reductase inhibitors, display pleiotropic immunomodulatory effects, independent of their lipid-lowering effects. The antiinflammatory effects of statins may contribute to their atheroprotective actions, and clinical trials are in progress to test whether these drugs have benefit in various autoimmune diseases. Published studies suggest that statins may

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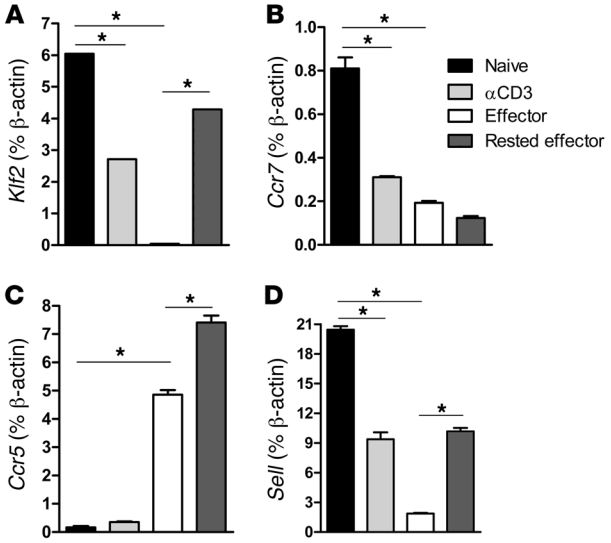

Figure 1
Changes in *Klf2* and chemokine receptor gene expression in T cells after stimulation. RNA was isolated from naive OT-1 CD8+ T cells, OT-1 cells after 6 hours of stimulation in vitro with αCD3, effector OT-1 cells generated by a 5-day culture, and effector T cells rested for 9 days after TCR stimulation in medium containing IL-2. (**A**) *Klf2*, (**B**) *Ccr7*, (**C**) *Ccr5*, and (**D**) *Sell* (*Cd62l*) RNA expression was determined by qRT-PCR analysis. Data represent the mean ± SEM of results from 3 experiments. *$P < 0.05$.

be beneficial for T cell–mediated diseases by suppressing inducible class II MHC expression and costimulators on APCs (11, 12), favoring Th2 versus Th1 differentiation of helper T cells (11, 13, 14), and augmenting circulating regulatory T cell numbers and their functional properties (15). However, the direct effects of statins on T cells remain poorly characterized. Statins are reported to bind to and block LFA-1 function, which is required for T cell interactions with APCs (16) and to block TCR signaling at Ras family GTPase-dependent steps, by interfering with prenylation of these signaling molecules (17, 18). Work with the human T cell leukemia line, Jurkat, suggests that statins may have antiproliferative effects on T cells independent of Ras, by uncoupling protein tyrosine kinases from TCR signal transduction pathways (19). An unproven hypothesis that has been put forward is that statins may impair T cell activation by altering membrane cholesterol levels and lipid raft formation, thereby perturbing the formation of the immune synapse between T cells and APCs (20). Statins upregulate KLF2 expression in endothelial cells (21, 22), and KLF2 expression is associated with a quiescent antiinflammatory phenotype of endothelial cells (23–25). In addition, there is a report that statins upregulate KLF2 expression in macrophages (26), and lentiviral-mediated KLF2 expression in macrophages mimics statin effects in reducing inflammatory chemokine expression (26). Taken together, these findings suggest that the beneficial effects of statins in treating cardiovascular disease include antiinflammatory actions independent of cholesterol lowering. We hypothesize that immunomodulatory effects of statins may also be related to their influence on T cell KLF2 expression. In the present study, we show that statins upregulate KLF2 expression in T cells and block activation-induced downregulation of T cell KLF2. Furthermore, statin treatment and exogenous *Klf2* gene expression have very similar effects on T cell functions and on the pathogenic actions of T cells in a mouse model of myocarditis.

Results

KLF2 expression pattern during T cell activation. We examined the expression of *Klf2* mRNA in TCR transgenic mouse CD8+ T (OT-1) cells before and after TCR stimulation using quantitative RT-PCR (qRT-PCR) (Figure 1A). *Klf2* was highly expressed in naive T cells, but it was rapidly downregulated after 6 hours of αCD3 stimulation. *Klf2* expression was very low to undetectable in mouse effector CD8+ T cells that were differentiated in vitro by a 5-day stimulation with αCD3, anti-CD28, and cytokines, but expression went up after 9 days of rest of the effector cells in IL-2–containing media. We observed a similar pattern of changes in *Klf2* mRNA expression in wild-type mouse CD8+ T cells and wild-type or TCR transgenic CD4+ T cells (data not shown). Expression of *Ccr7* and L selectin (*Sell*) mRNA was relatively high and *Ccr5* expression was relatively low in naive CD8+ T cells, and this pattern was reversed in effector T cells (Figure 1, B–D). These data, which show a positive correlation of *Klf2* expression with both *Ccr7* and *Sell* expression and an inverse correlation between *Klf2* and *Ccr5*, are consistent with published studies in various T cell populations (7, 9, 10), including KLF2-deficient T cells, which express more inflammatory chemokine receptors than wild-type T cells (9). Resting effector T cells expressed more *Klf2*, *Sell*, and *Ccr5* and less *Ccr7* than recently activated effector T cells (Figure 1, B–D).

Effects of statins on T cell KLF2 expression. We predicted that statins would prevent KLF2 downregulation after T cell activation or would upregulate KLF2 expression in activated T cells. These predictions were confirmed by analyzing *Klf2* mRNA in statin-treated OT-1 cells (Figure 2). The level of *Klf2* expression in naive CD8+ T cells was significantly reduced 6 hours after initiation of αCD3 stimulation but not when fluvastatin, lovastatin, or simvastatin, all at 10 μM, were present in the culture (Figure 2A). Pitavastatin also blocked downregulation of *Klf2* in naive OT-1 cells upon TCR stimulation, with a dose-dependent effect detectable from 0.5 to 10 μM (Figure 2B). We also examined the effect of statins on *Klf2* expression in effector CD8+ T cells, which have very low levels in the absence of statins. Treatment of effector CD8+ T cells for 18 hours with 10 μM fluvastatin, lovastatin, or simvastatin (Figure 2C) or with 1, 5, or 10 μM pitavastatin (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI41384DS1) significantly increased *Klf2* mRNA from low levels in vehicle-treated cells. Simvastatin treatment of effector OT-1 cells also increased *S1pr1*, *Ccr7*, and *Cd62l* expression (Figure 2D). Simvastatin treatment of naive and effector CD4+ T cells also resulted in elevated *Klf2* mRNA levels (Supplemental Figure 2, A and B). In order to determine whether in vivo exposure to statins can influence T cell *Klf2* expression, we performed 2 different types of experiments. First, we treated C57BL/6 mice with daily i.p. injections of 20 mg/kg lovastatin or vehicle (DMSO) for 3 consecutive days. Splenic CD8+ and CD4+ T cells were then purified and stimulated ex vivo with αCD3 in culture for 6 hours, before RNA isolation and qRT-PCR analysis. We found that resting CD8+ T cells from both control- and statin-treated mice had similar levels of *Klf2* mRNA, but after ex vivo anti-CD3 stimulation, the CD8+ T cells from lovastatin-treated mice had 6.5-times higher *Klf2* levels than the T cells from the control mice (Figure 2E). *Klf2* expression was also significantly higher after ex vivo stimulation in CD4+ T cells from lovastatin-treated mice com-

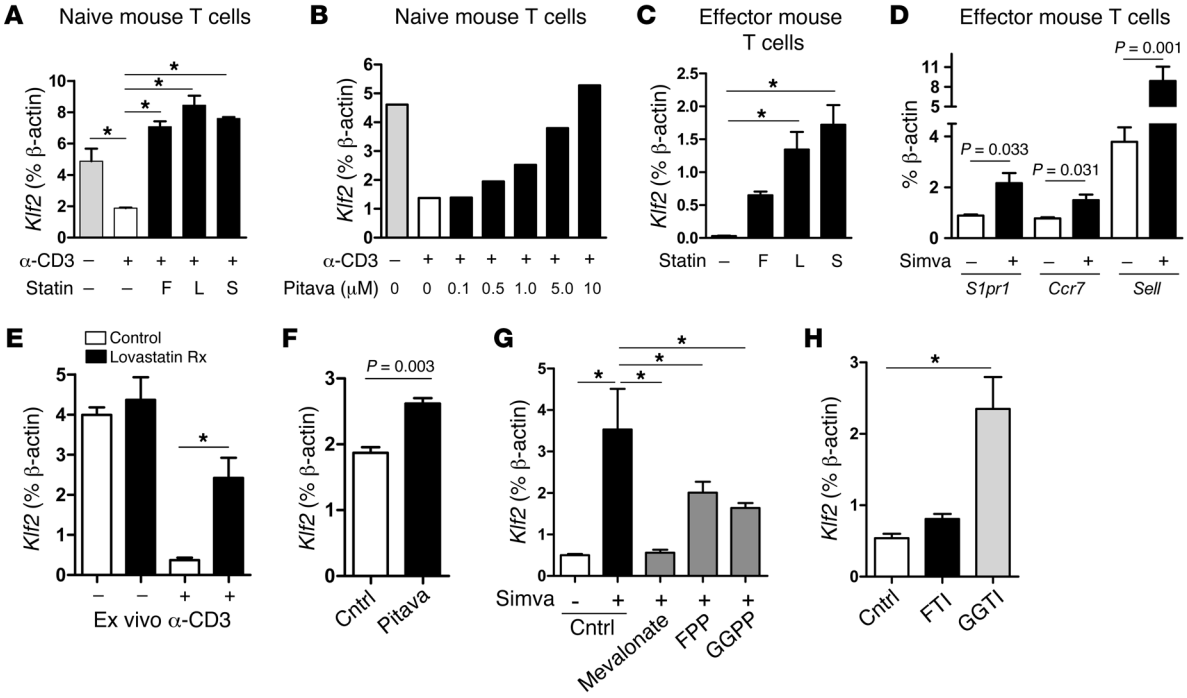

Figure 2

Statins increase mouse T cell *KLF2* expression by a mevalonate/isoprenoid-dependent pathway. (**A** and **B**) Naive OT-1 T cells were treated for 18 hours with vehicle (–), fluvastatin (F) (10 μM), lovastatin (L) (10 μM), or simvastatin (S) (10 μM), or pitavastatin (Pitava) at indicated concentrations and then stimulated with αCD3 for 6 hours before RNA isolation and qRT-PCR *Klf2* analysis. (**C** and **D**) OT-1 effector cells were treated with indicted statins for 18 hours, before RNA isolation and (**C**) qRT-PCR analysis of *Klf2* or (**D**) *S1pr1*, *Ccr7*, and *Sell*. (**E**) C57BL/6 mice were injected i.p. with 20 mg/kg lovastatin or DMSO for 3 days, and then splenic $CD8^+$ T cells were isolated and cultured with or without αCD3 for 6 hours, before RNA isolation and qRT-PCR analysis for *Klf2*. (**F**) OT-1/*Rag-2*$^{-/-}$ mice were fed pitavastatin (30 mg/kg) or vehicle twice daily for 2 days, immunized subcutaneously with ovalbumin/CFA, and fed pitavastatin (30 mg/kg) or vehicle for 2 more days before sacrifice, RNA isolation from draining lymph node T cells, and qRT-PCR *Klf2* analysis. (**G**) Effector OT-1 cells were cultured with vehicle, 10 μM simvastatin (Simva), 100 μM mevalonate, 10 μM farnesyl pyrophosphate (FPP), or 10 μM geranygeranyl pyrophosphate (GGPP), in indicated combinations for 8 hours, before RNA isolation and qRT-PCR analysis. (**H**) Effector OT-1 cells were cultured with vehicle, 20 μM FTI, or 20 μM GGTI for 8 hours, before RNA isolation and qRT-PCR analysis. (**A** and **C**–**H**) Data are mean ± SEM from 3 experiments. (**B**) Data are from 1 of 2 experiments performed, with similar results. *$P < 0.05$.

pared with control mice (Supplemental Figure 2C). We also tested whether statin treatment would affect *Klf2* expression in T cells after in vivo antigen-induced activation. In order to obtain enough in vivo antigen-activated T cells, we elected to subcutaneously immunize OT-1/*Rag2*$^{-/-}$ TCR transgenic mice with ovalbumin in complete Freund's adjuvant and to harvest draining lymph node T cells for RNA isolation and analysis. One hundred percent of the lymph node T cells in these mice were specific for ovalbumin, and a significant proportion of the draining lymph node T cells were activated by immunization, allowing for detection of changes in *Klf2* above the high *Klf2* levels found in the fraction of naive T cells that did not respond to the immunization. Using this protocol, we did find a significant increase in *Klf2* mRNA expression (Figure 2F).

Previous studies on endothelial cells and T cells have indicated that statins exert at least some of their biological effects by blocking the formation of isoprenoids through the mevalonate pathway (21, 27). We therefore tested whether the effects of statins on murine T cell *Klf2* expression were dependent on this pathway. We found that simvastatin-induced *Klf2* upregulation in effector T cells in vitro was completely blocked by the addition of mevalonate. The statin effect was approximately 50% blocked by either farnesyl pyrophosphate (FPP) or geranygeranyl pyrophosphate (GGPP) (Figure 2G). Furthermore, treatment of effector mouse T cells with a geranyl-geranyl transferase inhibitor (GGTI), but not a farnesyl transferase inhibitor, reproduced the statin effect of increasing *Klf2* expression (Figure 2H). These findings indicate that *Klf2* expression in T cells is suppressed by a geranylation-dependent pathway, and statins release this suppression by blocking synthesis of mevalonate, which is required for the formation of GGPP.

In order to determine whether statins can influence *KLF2* expression in human T cells, we isolated human blood $CD3^+$ T cells from healthy donors, activated them in vitro with αCD3 or phytohemagglutinin (PHA) plus IL-2, and treated the cells with vehicle or statins, before preforming RNA isolation and qRT-PCR analyses. We found that TCR stimulation reduces *KLF2* expression in the human T cells, but simvastatin or pitavastatin treatment blocks or reverses this reduction (Figure 3, A–C). We also examined the expression of KLF2 protein in control- and statin-treated human T cells using Western blot (Figure 3D). Both simvastatin and pitavastatin upregulated KLF2 protein expression in αCD3-stimulated T cells. We also determined, using qRT-PCR, that simvastatin increased human T cell expression of the complement regulatory molecule *CD59* (data not shown), which is a protein previously shown to be upregulated by statins in endothelial cells (28). The

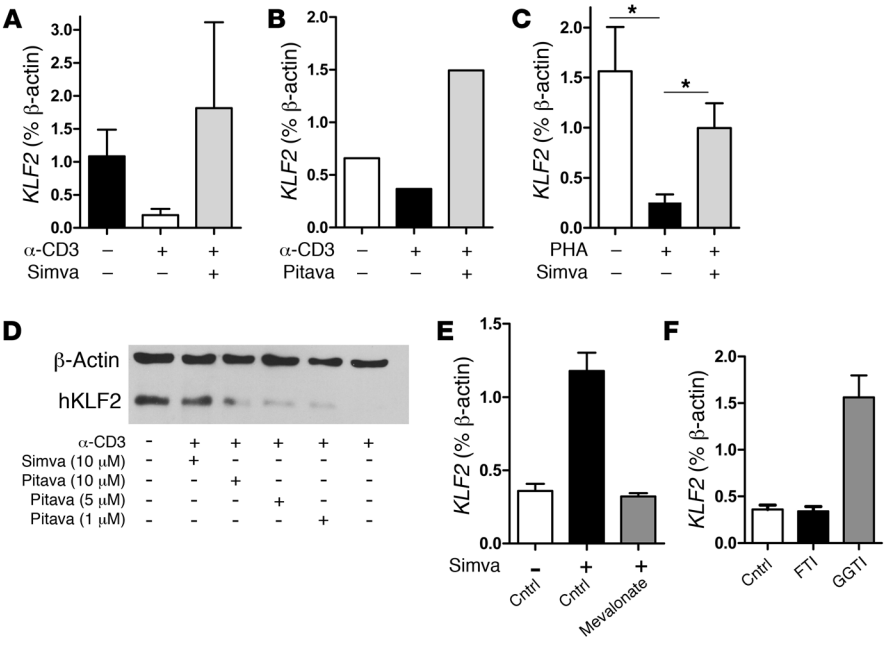

Figure 3
Statins increase human T cell *KLF2* expression by a mevalonate/isoprenoid-dependent pathway. Normal human blood T cells were stimulated with αCD3 (6 hours) or with IL-2 plus PHA for 4 days and/or either simvastatin or pitavastatin for 18 hours, before RNA or cytoplasmic lysate isolation, and (**A**–**C**) qRT-PCR analysis or (**D**) Western blot analysis for KLF2 expression was performed. Simvastatin was used at 10 μM and pitavastatin was used at 1 μM or indicated concentrations. (**E**) Normal human blood T cells were cultured with vehicle or 10 μM simvastatin alone or with 100 μM mevalonate for 8 hours, before RNA isolation and qRT-PCR analysis of *KLF2*. (**F**) Normal human blood T cells were cultured in the presence of vehicle, 20 μM FTI, or 20 μM GGTI for 8 hours, before RNA was isolated from the T cells for qRT-PCR analysis. (**A**) Data are the mean ± SD from 2 donors. (**B**) Data are from 1 of 2 donors, with similar results. (**C**) Data are the mean ± SEM from 3 different donors. *$P < 0.05$ (ANOVA). (**D**) Data are from 1 of 2 donors, with similar results. (**E** and **F**) Data are means ± SD of triplicate samples from 1 of 2 donors, with similar results.

addition of mevalonate also blocked the statin effect on human T cell *KLF2* expression (Figure 3E), and GGTI reproduced the statin effect (Figure 3F). These findings are similar to our findings with mouse T cells (Figure 2), indicating that human T cell *KLF2* expression is suppressed by an isoprenylation-dependent mechanism and that this mechanism is conserved between these 2 species.

Effects of statins on T cell functional responses. We next examined the influence of statins on functional responses of mouse and human T cells. We found that the proliferative responses of naive mouse CD8$^+$ T cells to TCR simulation were markedly inhibited by simvastatin or pitavastatin (Figure 4, A and B). We also observed that effector CD8$^+$ T cell proliferation (Figure 4C) and IFN-γ expression (Figure 4, D and E) were both markedly inhibited by statins. These data suggest that statins could impair both priming and effector phases of T cell responses. Simvastatin and pitavastatin also inhibited the modest proliferative responses of human blood T cells stimulated with αCD3 (Figure 4F) and the more robust proliferative responses of the human T cells to αCD3 plus anti-CD28 (Figure 4G). Human T cell *IFNG* expression, induced by PHA plus IL-2 stimulation, was also significantly reduced by simvastatin (Figure 4H).

Statin effects on T cell function are dependent on upregulation of KLF2. In order to directly test whether statin effects on T cell function are mediated through modulation of KLF2 expression, we chose to study the influence of statins on human T cell functional responses when *KLF2* expression was blocked. Reliable RNA knockdown in primary mouse T cells has proven very difficult to accomplish, and mice with T cell–specific deficiency of KLF2 have very few mature peripheral T cells (7, 9, 29). Therefore, in order to directly demonstrate that statin effects on mature effector T cell function are dependent on changes in *KLF2* expression, we chose to block *KLF2* translation in activated human T cells, using a lentiviral shRNA approach. We infected human blood T cells with lentiviruses expressing GFP and either *KLF2*-specific shRNA or random (control) shRNA. In order to accomplish efficient infection with shRNA-lentiviral vectors, it was necessary to first stimulate the cells with PHA plus IL-2. Since TCR stimulation markedly downregulated KLF2, we rested the T cells for 14 days before infection and statin treatment. As seen in Figure 5A, we found that statin induction of T cell *KLF2* was diminished by *KLF2* shRNA–expressing virus, compared with that of control virus. We were able to block *KLF2* expression by between 80%–92% in statin-treated human T cells, in 4 separate experiments. Importantly, we found *KLF2* shRNA–expressing virus completely blocked the ability of statins to reduce T cell *IFNG* expression (Figure 5B). Without shRNA knockdown, statin treatment reduced *IFNG* expression by 59%. The level of *IFNG* mRNA in *KLF2* shRNA–infected cells was markedly higher than that in control-infected cells, and statin treatment did not reduce the elevated level. Therefore, the suppression of *IFNG* by the statin was completely dependent on *KLF2* upregulation, establishing the necessity of KLF2 as a mediator of the T cell antiinflammatory effects of statins.

Statin treatment of CD8$^+$ effector T cells reduces their pathogenicity in vivo. Because we found that statins reduce CD8$^+$ functional responses, we predicted that CD8$^+$ T cell–mediated pathology would be abrogated by statin treatment of T cells. In order to investigate this possibility, we took advantage of a model of mouse of CTL-mediated myocarditis, previously developed in our laboratory, in which effector OT-1 cells (ovalbumin specific) are transferred into cMy-mOva mice, which express ovalbumin in cardiomyocytes (30, 31). Accordingly, we treated effector OT-1 cells with 10 μM simvastatin or DMSO for 18 hours, before their adoptive transfer into cMy-mOva mice. Aliquots of T cells were removed before adoptive transfer, and analysis of these cells confirmed that the expression of *Klf2* and *S1pr1* was upregulated and that of *Ifng* was downregulated by simvastatin, as we observed before (Figure 6A). Likewise, the proliferative responses of the statin-treated OT-1 cells to αCD3 were reduced (Figure 6B). FACS analysis indicated that both DMSO- and simvastatin-treated cells were equally well labeled with CFSE, and they were all viable (data not shown). Five days after transfer of statin or vehicle control–treated OT-1 cells, cMy-mOva

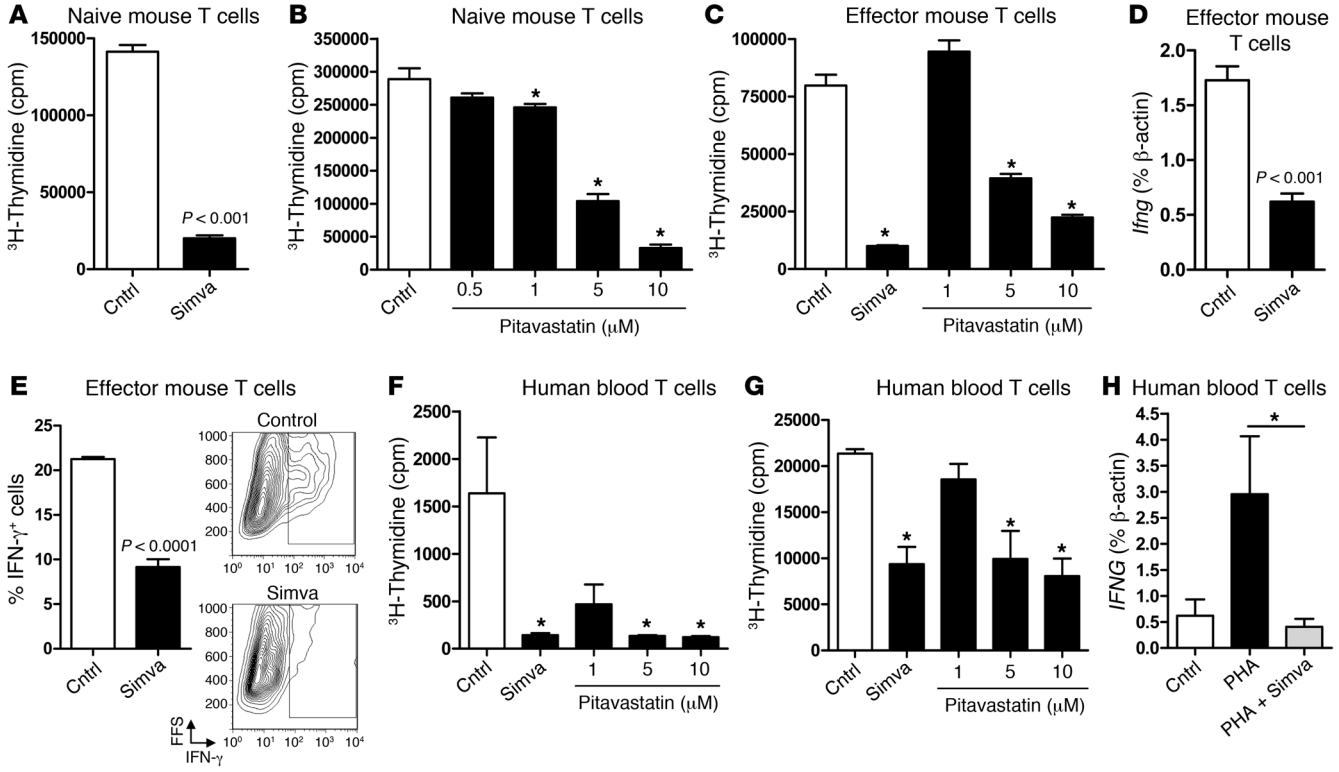

Figure 4

Statins reduce T cell proliferation and cytokine expression in vitro. (**A** and **B**) Naive or (**C**) effector mouse OT-1 cells were stimulated with αCD3 in the absence or presence of 10 μM simvastatin or the indicated concentrations of pitavastatin for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation during the final 16 hours of culture. (**D** and **E**) Effector mouse OT-1 cells were simulated with αCD3 in the absence or presence of simvastatin for 48 hours, and (**D**) RNA was isolated and analyzed by qRT-PCR for *Ifng* expression, or (**E**) the cells were analyzed for intracellular IFN-γ by immunofluorescence staining and flow cytometry. (**F**) Human blood CD3$^+$ T cells were stimulated with αCD3 or (**G**) mixture of αCD3, anti-CD28, and IL-2 for 48 hours in the absence or presence of 10 μM simvastatin or the indicated concentrations of pitavastatin, and proliferation was measured by ^{3}H-thymidine incorporation during the final 16 hours of culture. (**H**) Human T cells were stimulated with PHA and IL-2 for 4 days, and then treated with 10 μM simvastatin for 18 hours, and *IFNG* expression was analyzed by qRT-PCR. Data are the mean ± SEM of triplicate experiments. *P* values are compared with vehicle control–treated groups. **P* < 0.05. The FACS plot shown in **E** is representative, and the column chart shows mean ± SEM, of 12 separate analyses, with 2 different OT-1 preparations.

mice were sacrificed and analyzed. There was no significant difference in the recovery of CFSE-labeled cells from the spleens of mice receiving control- versus statin-treated T cells (Figure 6C), indicating equivalent viabilities over 5 days. The cardiac draining lymph node (CDLN), which we have previously described as a site reflecting cardiac inflammation (30, 31), was visibly larger in each of the mice that received control T cells compared with the nodes of the recipients of statin-treated T cells, and this was corroborated by total cell counts of the lymph nodes (7.7×10^7 vs. 2.1×10^7 cells/node in control vs. statin-treated groups, respectively). There were 2.3-times more vehicle control–treated OT-1 cells in the CDLNs than simvastatin-treated OT-1 cells (Figure 6D). There were also more B cells and CD11b$^+$ cells in the nodes of the control group, compared with the mice that received simvastatin-treated OT-1 cells (data not shown), indicative of more cardiac inflammation in the control group. There were more inflammatory cells in the myocardium of the mice receiving the control T cells compared with mice receiving simvastatin-treated T cells, as indicated by histopathological scoring (Figure 6E). Immunohistochemical analyses of the presence of transferred OT-1 cells in the myocardium revealed significantly more control T cells compared with simvastatin-treated T cells in the hearts of recipient mice (Figure 6F). Serum troponin levels, which are directly related to the degree of myocyte damage, were over 6-times higher in mice that received control- versus simvastatin-treated T cells (Figure 6G). qRT-PCR analysis of RNA from heart tissue revealed significantly more *Ifng*, *Vcam1*, *Cxcl10*, and *Ccl5* in the hearts from mice injected with control T cells compared with those injected simvastatin-treated T cells (Figure 6H).

In order to determine whether in vivo statin treatment could suppress pathogenic effects of T cells, we transferred OT-1 T cells into pitavastatin- or vehicle-treated cMy-mOva mice and sacrificed them 5 days after transfer. We found that cardiac damage, as assessed by serum troponin levels, was markedly reduced in the statin-treated mice (Supplemental Figure 3). This finding is consistent with direct statin effects on T cells but may also reflect effects on APCs.

Ectopic KLF2 expression in CD8$^+$ effector T cells mimics effects of statin treatment and reduces their pathogenicity in vivo. In light of the findings that statins increased the expression of Klf2 in OT-1 T cells and reduced their cardiac pathogenicity and that the statin effects are mediated, at least in part, through KLF2 expression, we predicted that ectopic expression of Klf2 would have similar effects as statin treatment. Accordingly, we prepared KLF2/GFP-expressing

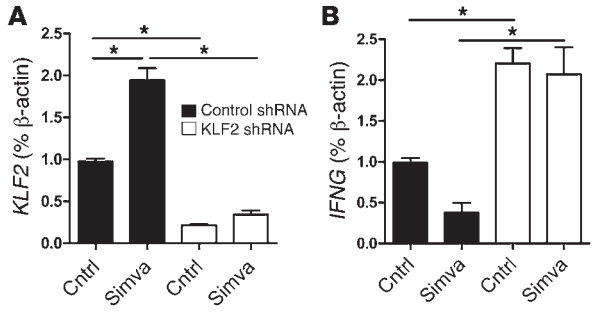

Figure 5

Blockade of *KLF2* mRNA expression impairs statin suppression of IFN-γ. IL-2– and PHA-stimulated human blood T cells were rested for 14 days and then infected with control shRNA/GFP– or *KLF2* shRNA/GFP–expressing lentivirus. GFP-positive cells, sorted 48 hours after infection, were further treated with or without 10 μM simvastatin for 18 hours and then analyzed using qRT-PCR for (**A**) *KLF2* or (**B**) *IFNG* expression. mRNA was quantified as the percentage of β-actin, and then the data were normalized for basal gene expression in rested, control virus–infected cells without simvastatin treatment. Data are mean ± SEM from 4 separate experiments. $^*P < 0.05$.

and control GFP–expressing retroviruses (KLF2-RV and Cntrl-RV, respectively), infected effector OT-1 cells with these viruses, FACS-sorted GFP$^+$ cells, and transferred them into cMy-mOva mice. *Klf2* mRNA expression in the KLF2-RV–infected T cells was about 6% of that of β-actin (*Actb*) mRNA levels, which is approximately 16-fold higher than in Cntrl-RV–infected T cells (Figure 7A). These levels, achieved by forced expression of ectopic Klf2, were about the same as those in naive OT-1 cells and statin-treated effector OT-1 cells (~4%–5% of *Actb* mRNA; see Figures 2, A, B, and Figure 6A). *S1pr1* mRNA levels were 3-fold higher in KLF2-RV–infected T cells compared with Cntrl-RV–infected T cells (Figure 7A). KLF2-RV–infected T cells also proliferated less in response to αCD3 than Cntrl-RV–infected T cells (Figure 7B). At the time of sacrifice, there was the same number of CFSE-labeled cells in the spleens of mice receiving Cntrl-RV– versus KLF2-RV–infected T cells (Figure 7C), indicating equivalent viabilities over 5 days. Five days after adoptive transfer, there were 8-times more Cntrl-RV–infected OT-1 cells in the CDLNs than KLF2-RV–infected OT-1 cells (Figure 7D). There were also more B cells and CD11b$^+$ cells in the nodes of the Cntrl-RV group compared with the KLF2-RV group (data not shown). Examination of the histological sections of cMy-mOva hearts revealed more inflammatory cell infiltration of the myocardia of the mice receiving the Cntrl-RV–infected T cells compared with mice receiving KLF2-RV–infected T cells (Figure 7E). Immunohistochemical analyses of the hearts from the cMy-mOva mice revealed significantly more OT-1 cells in mice receiving Cntrl-RV–infected T cells compared with mice receiving KLF2-RV–infected T cells (Figure 7F). Serum troponin levels were over 30-times higher in mice that received Cntrl-RV–infected versus KLF2-RV–infected T cells (Figure 7G). qRT-PCR analysis of RNA from heart tissue revealed significantly more *Ifng*, *Vcam1*, *Cxcl10*, and *Ccl5* expression in the hearts from mice injected with Cntrl-RV–infected T cells compared with those injected with KLF2-RV–infected T cells (Figure 7H). Overall, the effects of KLF2 overexpression in OT-1 cells, in the cMy-mOva myocarditis model, were remarkably similar to the effects of ex vivo statin treatment of the T cells shown in Figure 6.

Discussion

In this study, we show that statins directly modulate T cell function, in vitro and in vivo. We also show that statins upregulate the expression of KLF2 in T cells and the functional effects of statins on T cells are, at least in part, dependent on KLF2 expression. These findings are consistent with 2 independent lines of published work, showing that statins have inhibitory effects on T cells and ameliorate T cell–mediated autoimmunity (reviewed in refs. 32, 33) and that dynamic changes in KLF2 expression in T cells is tightly linked to activation status and migratory function of T cells (2, 6, 7, 10, 34). Previous work has also established that statins upregulate KLF2 expression in endothelial cells and that KLF2 expression is linked to an antiinflammatory phenotype of endothelium (21, 23).

Our studies establish direct effects of statins on T cell function in both mouse and human T cells and in both CD4$^+$ and CD8$^+$ T cells. Although statins have a variety of effects on myeloid APCs that may have significant impact on T cell activation, our in vitro experiments were conducted with polyclonal stimuli in the absence of APCs in order to discern direct T cell effects. A variety of lipophilic statin drugs showed similar effects. Others have shown direct effects of statins on T cells, and various mechanisms for these effects have been proposed, such as inhibition of isoprenylation of Rho/Rac family GTPases (33). We show here that the statin effect on T cell *KLF2* expression is completely abrogated by mevalonate, the metabolite generated by HMG-CoA reductase, and partially abrogated by isoprenoids, the downstream products of mevalonate. Our finding that statins induce expression of a gene that directly downregulates T cell activation has not been previously shown to our knowledge.

We examined effects of statins on T cell KLF2 expression and function in vivo in 4 different ways. First, we treated mice with statin injections over 3 days and then examined *Klf2* mRNA in T cells isolated from spleens. We found significant elevations in *Klf2* mRNA levels in the T cells ex vivo after αCD3 treatment. Second, we gavage treated ovalbumin-specific TCR transgenic OT-1 mice with statins or vehicle before and after subcutaneous ovalbumin immunization, and we determined that *Klf2* mRNA was upregulated in the draining lymph node T cells. Third, we treated effector OT-1 cells with vehicle or statins in vitro and then transferred them into cMy-mOva mice expressing ovalbumin in the myocardium. Using this established model of CD8$^+$ T cell–mediated cardiac inflammation (30, 31, 35), we found that statin treatment markedly reduced the T cell pathogenicity. Fourth, we gavage fed cMy-mOva mice vehicle or statins before and after transferring OT-1 cells and again found that cardiac damage was reduced in the statin-treated group. The basis for the reduced pathogenicity of statin-treated T cells could reflect reduced proliferative activity, altered homing functions, and reduced effector functions. We found no consistent changes in expression of chemokine receptors for inflammatory chemokines between statin-treated cells and control T cells (data not shown). Statin treatment did upregulate *Ccr7* and *Sell* mRNA, which encodes proteins that direct T cell homing to lymph nodes, but paradoxically, we found fewer statin-treated OT-1 cells in the heart draining lymph node than control T cells. Statin-treated OT-1 cells also had upregulated S1PR1. Because the S1PR1 is required for T cell efflux from lymph nodes, it is plausible that statin-treated effector T cells were more likely to enter the CDLN, become reactivated there, and exit the node. Nonetheless, they may have proliferated less in the nodes. Statin treatment also reduced IFN-γ expression by the T cells, but this is not likely explain reduced

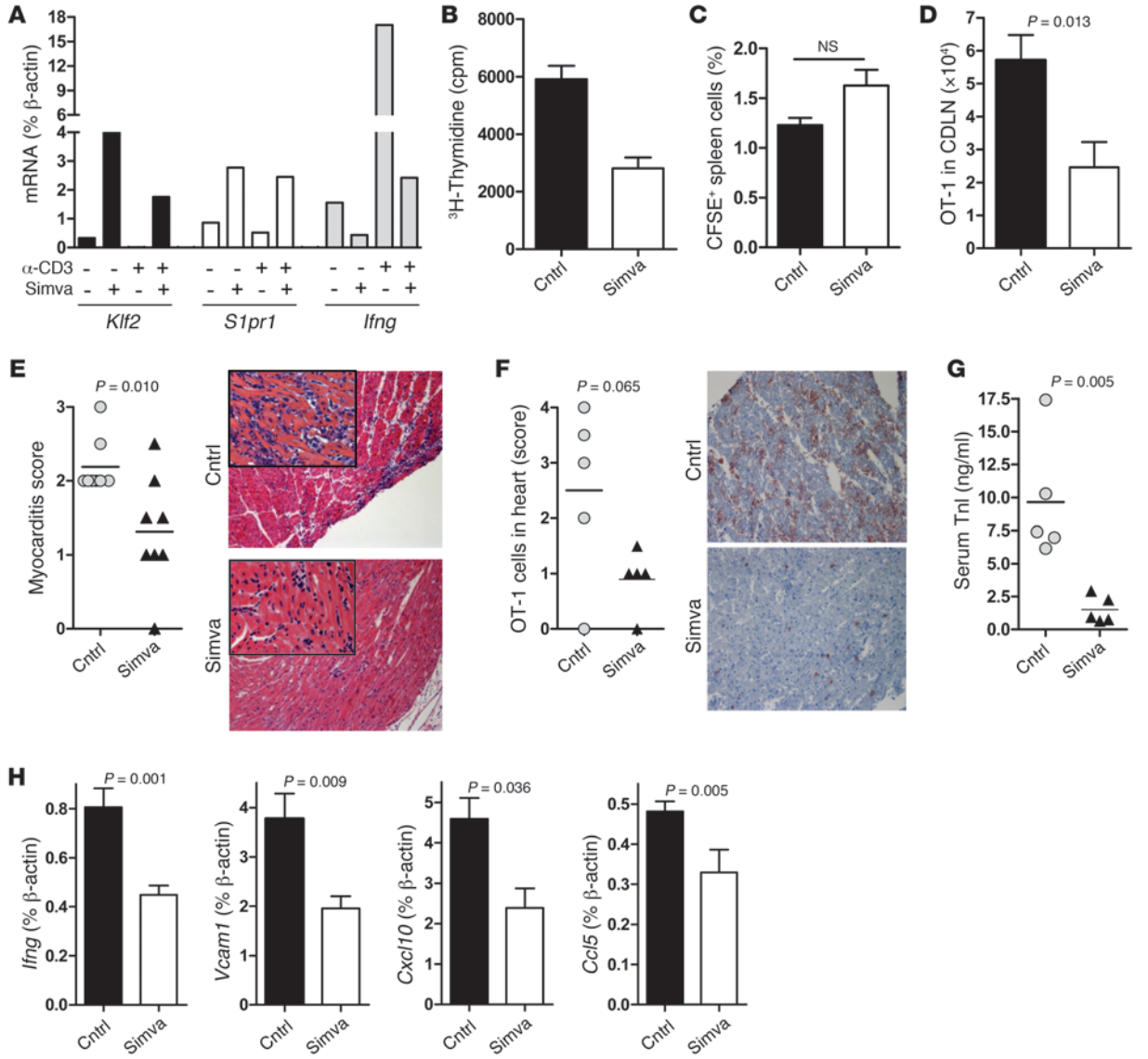

Figure 6

Statin treatment reduces pathogenicity of T cells. Effector OT-1$^{Thy1.1}$ cells were treated with DMSO (cntrl) or 10 μM simvastatin for 18 hours, and then left untreated or restimulated with αCD3 for 24 hours before either (**A**) performing qRT-PCR analysis of *Klf2*, *S1pr1*, and *Ifng* expression or (**B**) analyzing proliferative responses, as described in previous figures. Aliquots of the same DMSO- or simvastatin-treated OT-1$^{Thy1.1}$ cells were labeled with CFSE and 5 × 10^4 cells were adoptively transferred into cMy-mOva$^{Thy1.2}$ mice. At day 5, the mice were sacrificed, the number of OT-1 cells in (**C**) spleen and the (**D**) CDLN was determined by FACS, (**E**) myocardial inflammation was scored on histological sections of heart, and (**F**) the relative numbers of OT-1 cells in the myocardium were determined by Thy 1.1–specific immunohistochemistry. (**G**) Troponin I (TnI) levels were determined in serum collected from 5 mice of each group, at the time of sacrifice. (**H**) qRT-PCR analyses of *Ifng*, *Vcam1*, *Cxcl10*, and *Ccl5* were performed on myocardial RNA. (**A** and **B**) Data are from 1 of 2 OT-1 preparations, with similar results. (**B**) Data are mean ± SD of quintuplicate determinations. (**C**, **D**, and **H**) Data are the mean ± SEM of samples from 5–9 mice in total, from 2 separate experiments with different OT-1 preparations. (**E**–**G**) Horizontal bars represent the mean of each group. Original magnification, ×100; ×400 (insets).

pathogenicity, because we have found that T cell IFN-γ deficiency is not protective in our model of myocarditis (35).

Our data unequivocally demonstrate that statins can induce *KLF2* gene expression in mouse and human T cells. We show that statins block the reduction in *Klf2* expression that normally occurs when naive T cells are activated through the TCR, and they significantly increase the usually minimal expression of *Klf2* in effector T cells. Previously reported studies have shown that KLF2 has multiple profound effects on T cell function (6, 7, 9, 10, 34, 36). Overall KLF2 is required for the expression of genes that maintain the phenotype of naive T cells and resting memory T cells. If statin effects on T cell phenotype and function are dependent on the induced changes in KLF2 expression, then these effects should be at least partly recapitulated by constitutive expression of an exogenously introduced *Klf2* gene. In fact, when we infected OT-1 effector cells with a *Klf2*-expressing retrovirus and increased constitutive expression over 16 fold compared with control virus–infected cells (a similar level to simvastatin-treated OT-1 effector cells), the resulting

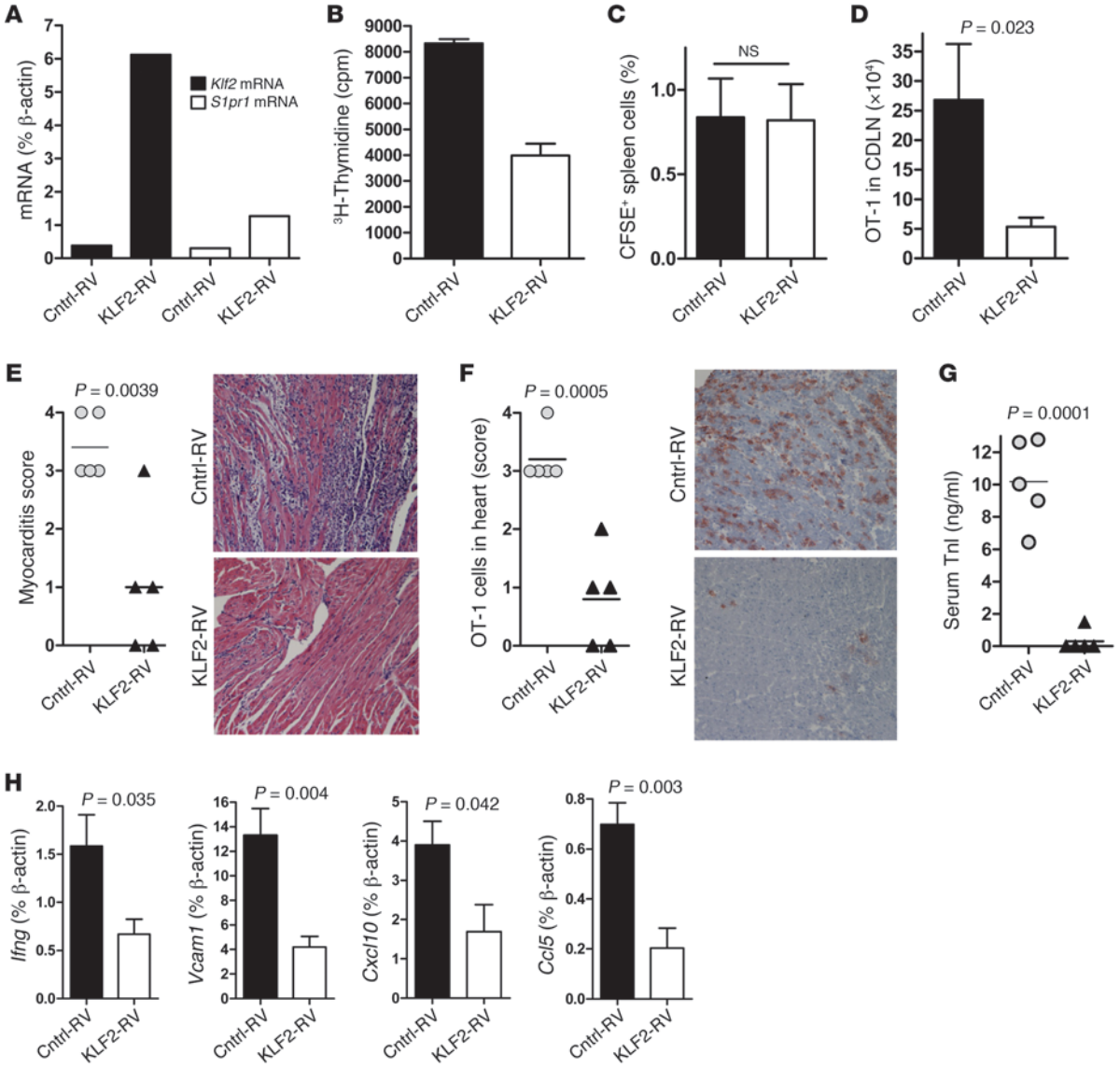

Figure 7

Ectopic KLF2 expression reduces pathogenicity of T cells. Effector OT-1$^{Thy1.1}$ cells infected with *Klf2*/GFP– or control GFP–expressing retroviruses (KLF2-RV or Cntrl-RV, respectively) were prepared, as described in Methods, and (**A**) analyzed using qRT-PCR for *Klf2* and *S1pr1* and (**B**) for proliferative responses to αCD3, as described in previous figures. Aliquots of the same KLF2-RV– or Cntrl-RV–infected OT-1$^{Thy1.1}$ cells (5×10^4) were adoptively transferred into cMy-mOva$^{Thy1.2}$ mice. At day 5, the mice were sacrificed and analyzed for OT-1 cells (**C**) in spleen and (**D**) in the CDLN, (**E**) the amount of myocardial inflammation, and (**F**) the relative numbers of OT-1 cells in the heart. (**G**) Serum troponin levels were determined, as described in Figure 6. (**H**) qRT-PCR analyses of *Ifng, Vcam1*, *Cxcl10*, and *Ccl5* were performed on myocardial RNA. (**A** and **B**) Data are from 1 of 2 experiments, with similar results. (**B**) Data are mean ± SD of quintuplicate determinations. (**C**, **D**, and **H**) Data are the mean ± SEM of samples from 5 mice in total. (**E**–**G**) Horizontal bars represent the mean of each group. Original magnification, ×100.

changes in the OT-1 cells were remarkably similar to the set of changes in OT-1 phenotype and function induced by statin treatment. The shared effects on OT-1 cells of ectopic KLF2 expression and statin treatment include elevated *S1pr1* expression, reduced proliferation, reduced numbers of cells in the CDLN after transfer into cMy-mOva mice, reduced inflammation and numbers of OT-1 cells in the hearts of these mice, and reduced inflammatory gene expression in the hearts.

We found that statins reduce IFN-γ expression in mouse and human effector T cells. In an in vivo model of graft arterial disease, statins were shown to decrease IFN-γ expression by T cells within the grafts (37). The ability of statins to reduce *IFNG* expression in each of 4 human T cell preparations was suppressed when *KLF2* upregulation was blocked, establishing a causal link between the statin effects on T cell function and on *KLF2* upregulation. We also observed increased *IFNG* gene expression in the human T cells in which KLF2 expression was blocked. Increased IL-4 but not IFN-γ expression was observed in KLF2-deficient mouse single-positive thymocytes (10). We did not detect *IL4* mRNA in either the control or the KLF2-blocked human T cells. Although we cannot explain the

apparent difference in the effects of KLF2 deficiency on cytokines between the mouse gene deletion study and our human knockdown study, our results are consistent with a role for KLF2 in regulating expression of effector cytokines by mature peripheral T cells.

In summary, statins reduce proliferative and functional responses of T cells and also induce higher levels of T cell KLF2 expression. These effects result in reduced pathogenicity of T cells in an in vivo model of $CD8^+$-mediated cardiac injury. The effects of statins on T cell function are dependent on KLF2 upregulation. Our results are important for the understanding of the therapeutic mechanisms of HMG-CoA reductase inhibitors and establish KLF2 as a potentially important target for the treatment of diseases in which proinflammatory T cells play a role, including atherosclerosis, myocarditis, and various autoimmune diseases.

Methods

Mice. Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. All other mice used were bred in the pathogen-free facility at the New Research Building (Harvard Medical School, Boston, Massachusetts, USA), in accordance with the guidelines of the Committee of Animal Research at the Harvard Medical School and the National Institutes of Health Animal Research Guidelines. All experiments performed with mice were approved by the Institutional Animal Care and Use Committee, Harvard Medical School. C57BL/6 cMy-mOva transgenic mice, which express membrane-bound ovalbumin exclusively on cardiomyocytes, were maintained on a C57BL/6 Thy1.2 (CD90.2) background and are referred to as cMy-mOva (30). The T cell–receptor transgenic OT-1 mouse strain, in which most T cells are $CD8^+$ and specific for Ova peptide 257–264 (SIINFEKL) bound to the class I MHC molecule $H\text{-}2K^b$, was maintained on a C57BL/6 Thy1.1 (CD90.1) or *Rag-1*$^{-/-}$ background.

T cell preparations, in vitro statin treatment, and CFSE labeling. Naive $CD4^+$ and $CD8^+$ T cells were purified using MACS beads from spleens of OT-1 or wild-type C57BL/6 mice, and effector T cells were generated by a 5-day in vitro culture with plate-bound αCD3, IL-2, IL-12 and anti-CD28, as described previously (31, 35). Some effector T cells were maintained for an additional 9 days in culture, with addition of IL-2, to derive resting effector cells. Human $CD3^+$ T cells were isolated using RosetteSep Human T Cell Enrichment Cocktail (Stem Cell Technologies) and activated with 4 μg/ml PHA (Sigma-Aldrich) and IL-2 (60 unit/ml) or αCD3 (BD Biosciences) and anti-CD28 (BD Biosciences). The use of human tissues for these experiments was approved by the Partners Human Research Committee, Boston, Massachusetts, USA, and informed consent was obtained for all blood donations. Some of the activated human T cells were kept in culture for an additional 2 weeks to derive resting effector cells. Lovastatin, fluvastatin, simvastatin, and pitavastatin were used in different experiments. All experiments included equal volume vehicle controls (ethanol, methanol, or DMSO). Before adoptive transfer, effector T cells, with or without statins treatment, were labeled with CFSE. Cell labeling and viability were examined by FACS analysis and trypan blue staining.

Reagents. Lovastatin, fluvastatin, and pitavastatin were obtained from Kowa Company Ltd. and diluted with ethanol before use. Simvastatin was purchased from Sigma-Aldrich and was diluted in DMSO. Mevalonate (Sigma-Aldrich), geranygeranyl pyrophosphate (Echelon Biosciences Inc.), and farnesyl pyrophosphate (Sigma-Aldrich) were diluted in DMSO. GGTI (GGTI-298) and farnesyl transferase inhibitor (FTI-277) (Sigma-Aldrich) were diluted in methanol.

In vivo statin treatment. OT-1/*Rag-1*$^{-/-}$ mice and cMy-mOva mice were fed pitavastatin (30 mg/kg) or vehicle twice daily by gavage feeding for 4 or 8 consecutive days, in 2 different experiments, before sacrifice and cell/tissue analyses (Figure 2F and Supplemental Figure 3). Alternatively, wild-type C57BL/6 mice were treated by i.p. injection of lovastatin (20 mg/ml) or vehicle for 3 consecutive days (Figure 2E and Supplemental Figure 2C).

Klf2-expressing retrovirus and shRNA-expressing lentivirus. A mouse *Klf2*-expressing retroviral vector was prepared by PCR-based cloning of mouse *Klf2* cDNA into a mouse stem cell virus–based vector and IRES2-EGFP that are expressed under the viral endogenous promoter. The virus was assembled in Phoenix cells provided by Garry Nolan (Stanford University, Stanford, California, USA), and viral supernatants were collected, filtered, and stored at –80°C. Naive OT-1 cells were activated with αCD3 and cytokine cocktails described above, and after 3 days, the KLF2-GFP or control-GFP virus was added to the cultures with polybrene (7.5 μg/ml). Human $CD3^+$ T cells were stimulated by IL-2 and PHA and then underwent resting for 14 days in culture. Cells were infected with human lentiviral shKLF2-GFP and control vector at a titer of MOI = 0.05. The lentiviral vector used was a modified LentiLox 3.7, targeting a *KLF2* sequence spanning from 1,484 to 1,501 (TTTGTACTGTCTGCGGCA) of the human cDNA. The virus was packaged using a HEK293FT cell line, and viral particles were isolated by ultracentrifugation and titered by FACS analysis performed on infected cells using GFP. Forty-eight hours after infection, both the mouse OT-1 cells and human T cells were sorted for positive-infected cells by FACS sorting. The sorted cells were used for different experiments.

Flow cytometry analyses and intracellular cytokine staining. For flow cytometric analysis of surface markers and cytoplasmic cytokines, cells were stained with directly conjugated fluorescence antibodies as described (30, 31) and analyzed with a FACSCalibur instrument (BD Biosciences) and FlowJo software (Tree Star). For intracellular staining, cells were restimulated for 4 hours at 37°C with 10 ng/ml PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), and 1 μM brefeldin A (eBioscience), and subsequently fixed with 1% formaldehyde for 15 minutes. After washing, cells were suspended with Perm/Wash TM buffer (BD Biosciences), before staining with conjugated fluorescence antibody against IFN-γ. Fluorochrome-conjugated, mouse-specific monoclonal antibodies purchased from BD Pharmingen, used for flow cytometry, included CD8 (clone 53-6.7) and CD90.1/Thy 1.1 (clone OX-7). Anti–IFN-γ (XMG 1.2) was purchased from Biolegend.

Cell proliferation assays. Murine and human T cells were cultured in αCD3-coated 96-well plates for 72 hours. In some experiments, human T cells were also stimulated with PHA (4 μg/ml) plus IL-2 (60 unit/ml). During the last 16 hours, cells were pulsed with 1 μCi of [^{3}H]thymidine (PerkinElmer), followed by harvesting and analysis of incorporated [^{3}H]thymidine in a β-counter (1450 Microbeta, Trilux; PerkinElmer).

Myocarditis studies with adoptively transferred OT-1 cells. Fifty thousand effector OT-1 cells were resuspended in PBS and injected i.p. into cMy-mOva mice, as described previously (31, 35, 38). In some experiments, the effector cells were labeled with CFSE before transfer. In other experiments, the OT-1 cells were infected with GFP-expressing retroviral vectors during differentiation from naive precursors, and GFP^+ cells were FACS purified before transfer into cMy-mOva mice. Five days after T cell transfer, cMy-mOva mice were euthanized by CO_2 inhalation. The spleens and CDLNs were removed for FACS analysis. After perfusion, the heart was surgically removed, and sections were frozen for immunohistochemistry, fixed in formalin for paraffin embedding and H&E staining, or processed for RNA extraction RT-PCR, as described previously (35, 38). Myocarditis was graded by microscopic examination of H&E-stained sections, performed in a blinded fashion by a trained pathologist, as described previously (31, 35, 38). Blood was collected from mice at time of sacrifice; serum levels of cardiac troponin-I were measured by a clinical quantitative immunoassay technique (TnI-Ultra, Siemens).

qRT-PCR analysis. Total RNA were extracted from cultured T cells or myocardium using TRIzol (Invitrogen Life Technologies) or the Rneasy kit (QIAGEN Inc.), reverse-transcribed using the ThermoScript RT-PCR system and random hexamer primers according to the manufacturer's instructions

(Invitrogen), and amplified by real-time PCR with SYBR Green PCR mix (Applied Biosystems) and an iCycler iQ Real-Time PCR Detection System (Bio-Rad) or Step-One Detection System (Applied Biosystems), according the manufacturer's instructions. Levels of specific gene expression in the samples are presented relative to endogenous levels of β-actin housekeeping gene expression in the same sample to normalize for mRNA differences between samples. The sequences of the primers are shown in Supplemental Table 1.

Western blot analysis. Total lysates from vehicle control– or statin-treated human $CD3^+$ T cells were immunoblotted and probed with mouse monoclonal anti-human KLF2 monoclonal antibody previously described (39). HRP-conjugated horse anti-mouse IgG (Sigma-Aldrich) was used to identify sites of binding of the primary antibody. After probing with the primary antibody, membranes were stripped of bound immunoglobulins and reprobed with anti–β-actin (Sigma-Aldrich).

Immunohistochemical staining. Immunohistochemical staining was performed on frozen acetone-fixed sections of heart tissue, as described previously (31, 35, 38). Specific antibodies used for immunohistochemistry include anti-CD90.1 (Thy1.1; BD Pharmingen,). Isotype-matched antibodies were used as controls.

Statistics. Statistical analyses were performed using the 2-tailed Student's *t* test or Mann-Whitney test, for experiments comparing 2 groups, and ANOVA with Tukey's Multiple Comparison post test, for 3 or more group experiments. *P* values of less than 0.05 are considered significant.

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Address correspondence to: Andrew H. Lichtman, Brigham and Women's Hospital, NRB Room 752N, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA. Phone: 617.525.4335; Fax: 617.525.4333; E-mail: alichtman@rics.bwh.harvard.edu.

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