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Aberrant expression of costimulatory molecules in splenocytes of the mevalonate kinase-deficient mouse model of human hyper-IgD syndrome (HIDS)

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Abstract

Objective—We sought to determine the activation status and proliferative capacities of splenic lymphocyte populations from a mevalonate kinase-deficient mouse model of hyper-IgD syndrome (HIDS). We previously reported that murine mevalonate kinase gene ablation was embryonic lethal for homozygous mutants while heterozygotes ($Mvk^{+/-}$) demonstrated several phenotypic features of human HIDS including increased serum levels of IgD, IgA, and TNFα, temperature dysregulation, hematological abnormalities, and splenomegaly.

Methods and results—Flow cytometric analysis of cell surface activation markers on T and B lymphocytes, and macrophage populations, demonstrated aberrant expression of B7 glycoproteins in all splenic cell types studied. Differences in expression levels between $Mvk^{+/-}$ and $Mvk^{+/+}$ littermate controls were observed in both the basal state (unstimulated) and after Concanavalin A (Con-A) stimulation in vitro of whole splenocyte cultures. In $Mvk^{+/-}$ CD4 and CD8 T cells, alterations in expression of CD25, CD80, CD152, and CD28 were observed. $Mvk^{+/-}$ splenic macrophages expressed altered levels of CD80, CD86, CD40, and CD11c while $Mvk^{+/-}$ B lymphocytes had differential expression of CD40, CD80, and CD86. $Mvk^{+/-}$ splenocyte subpopulations also exhibited altered proliferative capacities in response to in vitro stimulation.

Conclusion—We postulate that imbalances in the expression of cell surface proteins necessary for activation, proliferation, and regulation of the intensity and duration of an immune response

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may result in defective T cell activation, proliferation, and effector functions in our model and potentially in human HIDS.

Hyper-immunoglobulin D (IgD) syndrome (HIDS) is a human inherited autoinflammatory disorder that arises from mutations in the mevalonate kinase gene (MVK), and is associated with recurrent inflammatory episodes (Goldfinger 2009). HIDS has been classified as a primary immunodeficiency disease (Notarangelo et al. 2009). Autoinflammatory disorders usually result from mutations in proteins that are important to functions of the innate arm of the immune system that ultimately affect the efficiency of T and B lymphocyte adaptive immune responses.

T cell activation, proliferation, and T helper (TH) cell subset differentiation (TH1, TH2) are functions of the immune response that are dependent on T cell receptor (TCR)-antigen/major histocompatibility antigen (MHC) recognition and costimulatory signals. Multiple costimulatory signals are critical for the activation and differentiation of naïve T cells and consist of interactions between T cells and antigen presenting cells (APCs), and also T cell-T cell interactions (Salomon and Bluestone 2001; Greenwald et al. 2005). A major costimulatory pathway involves interactions of the B7 family of glycoproteins expressed on lymphocytes, macrophages, dendritic cells, and tissues involved in antigen presentation in disease states. Expression of B7 glycoproteins in this study included CD28, CD152 (CTLA-4), CD80 (B7-1) and CD86 (B7-2). The B7 ligands CD80 and CD86 are expressed constitutively at low levels in resting APCs and T cells and are upregulated during activation. It is believed that basal constitutive expression may limit T cell activation, thereby maintaining self-tolerance, and also maintaining a population of T regulatory cells (Treg) (Lohr et al. 2003).

CD28 is constitutively expressed on naïve T cells and is rapidly upregulated within hours of activation (Freeman et al. 1993). CD152 expression on T cells is very low in naïve T cells but is upregulated within 24–48 hours of T cell activation in vitro (Perkins et al. 1996). Both CD28 and CD152 bind CD80 and CD86 but with differing affinities. CD152 binds CD80 with greater affinity than CD86 (Freeman et al. 1993). CD28 interaction with CD80 or CD86 provides a strong stimulatory signal to mature T cells that have been activated through the T cell receptor (TCR). Moreover, CD28 ligation induces IL-2 production and upregulation of surface CD25, the IL-2 receptor α chain, and proliferation (Jenkins et al. 1991; Butscher et al. 1998). It has been suggested that CD28-CD86 interactions are more important for initial T cell activation while CD28-CD80 interactions are important for sustained T cell activation (Sethna et al. 1994).

CD152 ligation induces an inhibitory signaling pathway that decreases IL-2 synthesis and inhibits T cell responses including proliferation by restricting cell cycle progression from G1 to S phase (Krummel and Allison 1996). CD152 may play a role in peripheral T cell tolerance through maintenance of a Treg population that is dependent on CD152-CD80 interactions (Lohr et al. 2003; Perez et al. 2008). Together, the interactions of B7 glycoproteins on cell surfaces function to regulate the intensity and duration of T cell activation and therefore, the outcome of immune responses involved in transplantation, antitumor immunity, autoimmunity, infectious diseases, and asthma and allergy (Blazar et al. 1999).

In the current study, we postulated that because HIDS patients experience chronic bouts of fever and autoinflammation, it is possible that their peripheral lymphocyte subpopulations may exhibit imbalances in cell types, activation states, cytokine responses, and proliferative capacities while suppressing chronic inflammation to achieve homeostasis. To test this

hypothesis, we first compared cell surface activation marker status of lymphocytes and macrophages from wild type and mevalonate kinase-deficient mice.

Materials and methods

Animals

Mvk mice were bred and housed under specific pathogen-free conditions under IACUC-approved protocols. Male mice~30 weeks of age were used in five experiments in these studies since the HIDS-like phenotype is more pronounced in aged animals (Hager et al. 2007). Age-matched female mice were used in one experiment that measured proliferation, B7 expression, and Foxp3 expression.

Splenocyte isolation

For each experiment, spleens were removed from 5 $Mvk^{+/+}$ and 5 $Mvk^{+/-}$ mice. Spleens were pooled according to genotype in order to have sufficient numbers of cells to simultaneously assess 44 markers at four different time-points in culture. Genotypic pooling of cells also offset variances in individual phenotypes. Splenocytes were obtained from intact spleens and red blood cells removed as described (McAdam et al. 2000). Cells were divided into two portions. One cell aliquot was used for basal cell surface marker immunostaining. The second cell aliquot was cultured in the presence of the T cell mitogen, Con-A, to induce direct stimulation of cell surface marker expression on activated T cells or, indirect induction of markers in other splenocyte populations through T cell cytokines and T cell-cell interactions.

Con-A splenocyte stimulation

Splenocytes were seeded at 5×10^5 cells per well of 96-well tissue culture plates and were stimulated with Con-A (2.5 μg/mL) for 24, 48, and 72 hours.

Flow cytometry

One million cells were stained directly with four fluorochrome-conjugated cell marker-specific antibodies, followed by fixation in 2% paraformaldehyde and analysis by FACSCalibur (BD Biosciences). Expression of a particular cell marker in basal (unstimulated, freshly-isolated) and activated (Con-A stimulated) splenocyte populations was assessed by measurement of the cell surface marker density indicated by mean fluorescent intensity (MFI) and the % positive of splenocytes staining with the respective marker. For analysis of forkhead box P3 (Foxp3), Con-A activated CD4 T cells were treated with reagents provided in the Foxp3 staining buffer set and fluorochrome-labeled anti-Foxp3 antibody (eBioscience). Isotype staining controls were performed to validate the specificity of all antibodies used for immunostaining.

T cell isolation, anti-CD3ε/anti-CD28 costimulation culture, and MTT proliferation assay

T cells were isolated from whole splenocyte preparations by magnetic bead negative selection using the CD4 T cell isolation kit (Miltenyi Biotec). Purified CD4 T cell populations (> 90% purity by flow cytometry) were collected and used for cell proliferation experiments. Anti-CD3 ε and anti-CD28 antibodies (BD Biosciences), at concentrations of 0.1 μg/mL and 1 μg/mL, respectively, were employed with overnight incubation. The following day, purified T cells were added and the plates were incubated at 37°C for up to 96 hours. Plates were prepared with six replicate wells per time-point for each genotype. Colorimetric quantification of cell proliferation was performed with the cell proliferation kit I (MTT) (Roche Applied Science). At each time-point (24, 48, 72, and 96 hours), fold-differences were determined for stimulated cultures compared to unstimulated cultures.

5-, 6-Carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling of splenocytes

Cells were labeled with 5 μM CFSE (Invitrogen; Kearney et al. 1994) for 15 minutes, followed by plating with Con-A. At harvest, replicate wells were pooled at each time-point (48, 72 hours). One million cells per sample were then stained with fluorochrome-labeled antibodies to specific cell surface lineage markers in order to determine the number of cell divisions of CD4 T cells, CD8 T cells, B cells, and macrophages.

Study design and statistical analysis

Two preliminary screenings of numerous cell surface markers described in Table 1 in male splenocyte populations revealed reproducible directional differences in certain cell marker expression in $Mvk^{+/-}$ mice as compared to $Mvk^{+/+}$. In three subsequent experiments, results were combined for each cell marker queried (not all markers were queried in every experiment due to insufficient cell numbers at harvest) and the differences between mean values ± SEM for each experimental group were analyzed using a one-tailed Student's *t* test for significance ($p<0.05$) since we had observed directionality in data collected in two preliminary staining experiments. The MFI data is displayed as bar graphs that show mean marker expression between multiple experiments which, in total, consisted of 10–20 mice of each genotype. The data were analyzed using GraphPad Prism 5 software.

Results

Expression levels of cell markers in basal and Con-A stimulated splenic CD4 T lymphocytes

CD4 T lymphocytes expressed all T cell markers listed in Table 1 similarly between $Mvk^{+/-}$ and $Mvk^{+/+}$ mice with the exception of the surface densities of CD4; the B7 glycoproteins CD80, CD152 and CD28; and CD25. Basal CD4 densities were consistently ~ 2-fold lower in $Mvk^{+/-}$ as compared to $Mvk^{+/+}$ (Fig. 1) although the percentages of CD4 cells were similar between genotypes (39.6 +/− 3.7% and 32.6 +/− 2.5%, respectively). CD4 surface densities remained similar to basal levels in Con-A stimulated culture although there was an approximate 10% increase in percentages of CD4 T cells in both genotypes at 72 hours.

Altered expression levels of B7 glycoproteins were observed in CD4 T cells in $Mvk^{+/-}$ mice. Basal levels of CD80 were consistently ~ 2-fold higher in $Mvk^{+/-}$ than $Mvk^{+/+}$ (Fig. 2a) indicating that these cells may be in a more activated state than basal $Mvk^{+/+}$ CD4 T cells (Salomon and Bluestone 2001; Taylor et al. 2004). Additionally, the basal density of CD152, the CD80/CD86 inhibitory receptor, was significantly elevated in $Mvk^{+/-}$ CD4 T cells ($p=0.0169$, Fig. 2b), a further indication of an activated T cell phenotype (Perkins et al. 1996; Allegre et al. 2006). CD152 levels were also ~ 2-fold higher in activated $Mvk^{+/-}$ versus $Mvk^{+/+}$ CD4 T Con-A stimulated cell cultures, with CD152 levels peaking in both genotypes at 24 hours. In these experiments, increased $Mvk^{+/-}$ CD152 levels coincided with decreased expression of CD25 (the IL-2 receptor α chain) in Con-A stimulated cultures ($p = 0.0108$, Fig. 2c) and decreased IL-2 accumulation (unpublished observation). These observations are consistent with CD152 ligation inhibiting T cell growth (Krummel and Allison 1996). Unlike CD152, basal levels of CD28, the CD80/CD86 stimulatory B7 receptor, were similar in both genotypes in CD4 T cells, but were consistently elevated by ~ 2-fold at 72 hours in activated $Mvk^{+/-}$ CD4 T cells (Fig. 2d) although IL-2 levels were decreased which is opposite to what would normally be expected. These results suggest that in *Mvk*-deficient CD4 T cells, CD152 mediated inhibitory regulation of T cell activation may occur to a greater degree than that of $Mvk^{+/+}$.

Enhanced ligation of CD152 by an agonistic antibody induces IL-10 and TGF-β resulting in expansion of the anti-inflammatory Foxp3 Tregs (Vasu et al. 2004; Li et al. 2007), and

blockade of the mevalonate pathway via statin administration results in generation of Foxp3 Treg cells (Kim et al. 2010). Based on these observations, we stained for intracellular levels of Foxp3 in CD4 T cells. In this experiment, we observed a significant increase in Foxp3% positive $Mvk^{+/-}$ CD4 T cells compared to $Mvk^{+/+}$ (p=0.0246, data not shown) suggesting that $Mvk^{+/-}$ mice have mobilized this Treg subset. Taken together, we postulate that blockade of mevalonate kinase in CD4 T cells results in alteration of B7 glycoprotein surface expression which may perturb signaling in the T-APC immunological synapse, favoring increased inhibition through CD152 and recruitment of $Foxp3^{+}$Tregs.

Expression levels of cell markers in basal and Con-A stimulated splenic CD8 T lymphocytes

Unlike CD4, the CD8 lineage marker for cytotoxic T cells was expressed in $Mvk^{+/+}$ and $Mvk^{+/-}$ T cells at similar basal densities (10,337±1,722 MFI and 8,690±893 MFI, respectively) and percentages of the splenocyte population (10.1± 1.2% and 10.1±1.4%, respectively). Similar to $Mvk^{+/-}$ CD4 T cells, $Mvk^{+/-}$ CD8 T cells consistently expressed ~ 2-fold higher basal levels of CD152, but unlike $Mvk^{+/-}$ CD4 T cells, activation-induced CD152 expression was similar between genotypes in CD8 T cells. CD80 basal expression was significantly decreased in $Mvk^{+/-}$ CD8 T cells (p = 0.0046, Fig. 3a) whereas it was consistently increased in $Mvk^{+/-}$ CD4 T cells (Fig. 2a). Additionally, CD80 expression was consistently decreased ~ 2-fold in activated $Mvk^{+/-}$ CD8 T cells, whereas CD80 expression in activated $Mvk^{+/-}$ CD4 T cells was similar to that of $Mvk^{+/+}$. $Mvk^{+/-}$ CD8 T cells consistently expressed ~ 2-fold lower basal levels of CD28 (Fig. 3b) while activated levels of CD28 were similar between genotypes unlike that of $Mvk^{+/-}$ CD4 T cells. CD25 basal levels were similar between genotypes in CD8 T cells as was observed in CD4 T cells. After 72 hours in Con-A stimulated cultures, $Mvk^{+/-}$ CD25 levels were significantly decreased (p=0.0364, Fig. 3c) similar to $Mvk^{+/-}$ CD4 T cells.

Expression levels of macrophage markers in basal and Con-A stimulated splenic cultures

Macrophages were identified based on expression of F4/80 antigen, a marker of mature murine macrophages. Of the macrophage markers listed in Table 1, male $Mvk^{+/-}$ manifested altered basal expression of CD80, CD86, CD40, and CD11c. Basal levels of $Mvk^{+/-}$ CD80 and CD86 were significantly decreased (p=0.0387, Fig. 4a) and (p=0.0291, Fig. 4b.), respectively. Basal expression levels of $Mvk^{+/-}$ macrophage CD40 were similar to $Mvk^{+/+}$; however, CD40-% positive macrophages in the $Mvk^{+/-}$ basal population were significantly decreased as compared to that of $Mvk^{+/+}$ (p=0.0098, Fig. 4c). In Con-A stimulated splenocyte cultures, the number of $Mvk^{+/-}$ CD40-% positive macrophages reached that of $Mvk^{+/+}$ suggesting that CD40 upregulation is not intrinsically impaired in $Mvk^{+/-}$ macrophages. Lastly, basal CD11c-% positive $Mvk^{+/-}$ macrophages were significantly increased as compared to $Mvk^{+/+}$ splenic populations (p=0.00487, Fig. 4d). It is noteworthy that CD11c is an integrin subunit of the complement iC3b and fibrinogen receptor associated with the macrophage M1 phenotype which is proinflammatory (producing TNFα, MCP-1, IL-1β,-6, -8, -10) (Wentworth et al. 2010). Together, these results indicated that $Mvk^{+/-}$ macrophages were deficient in CD80, CD86, and CD40 expression in the basal state but were responsive to signaling induced in stimulated mixed splenocyte culture to increase expression of these markers to normal levels.

Expression levels of B lymphocyte markers in basal and Con-A stimulated splenic culture

B lymphocytes were identified based on the presence of CD19 and surface IgM and IgD. Basal level expression of the B cell markers listed in Table 1 were normal in $Mvk^{+/-}$ as compared to $Mvk^{+/+}$ B cells except for a consistent ~ 2-fold decrease in CD40-% positive $Mvk^{+/-}$ B cells similar to the observed basal state of $Mvk^{+/-}$ macrophages. Like the other $Mvk^{+/-}$ splenic cell types we studied, $Mvk^{+/-}$ B cells also had altered expression of B7

glycoproteins, particularly in Con-A stimulated splenic cultures. Stimulated $Mvk^{+/-}$ B cells expressed significantly increased levels of CD80 (p=0.0017, Fig. 5a) as compared to $Mvk^{+/+}$ B cells. Increased CD80 levels were concomitant with significantly decreased expression of CD86 in $Mvk^{+/-}$ B cells (p = 0.0243, Fig. 5b) in Con-A stimulated splenocyte cultures. CD86-% positive B cells were also significantly decreased (p=0.0133, Fig. 5c) in these cultures. These data suggest that CD80-CD152 inhibitory signaling may predominate at the T-B cell immunological synapse in $Mvk^{+/-}$ mice.

Gender differences in CD80 expression and proliferation of splenocyte subpopulations

CD80 is expressed abnormally in both $Mvk^{+/-}$ female and male splenocytes, though oppositely. Basal female $Mvk^{+/-}$ macrophage and B cell CD80 levels were expressed at~2-fold higher levels than $Mvk^{+/+}$ females. Recall that in $Mvk^{+/-}$ males, basal CD80 levels were significantly decreased in macrophages (Fig. 4a). During Con-A stimulation $Mvk^{+/-}$ male macrophages expressed CD80 at levels similar to $Mvk^{+/+}$. This was not observed in $Mvk^{+/-}$female macrophages and B cells. In females, CD80 expression was ~ 3-fold higher in macrophages and ~ 6-fold higher in B cells in splenocyte Con-A stimulated cultures as compared to expression in $Mvk^{+/+}$. $Mvk^{+/+}$ females also had increased CD80 expression following Con-A stimulation as compared to $Mvk^{+/+}$ males with macrophage CD80 levels ~ 4-fold higher and B cell levels ~ 2-fold higher. Curiously, there were no notable differences between genders in any of the markers queried (Table 1) with the exception of CD80.

To determine if splenic subpopulations had altered proliferative capacities, we performed experiments in which cultured male splenocytes were labeled with CFSE in order to assess cellular proliferation. $Mvk^{+/-}$ CD4 and CD8 T cells both increased in number by ~ 2-fold greater than those of $Mvk^{+/+}$; $Mvk^{+/-}$ B cells had increases ~ 3-fold greater than those of $Mvk^{+/+}$ B cells in number; and the number of $Mvk^{+/-}$ macrophages had increased ~ 5-fold greater than those of $Mvk^{+/+}$. We also purified CD4 T cells from male mice and stimulated growth in vitro by anti-CD28/anti-CD3ε costimulation. We observed that similar to CD4 T cells in Con-A stimulated splenocyte cultures, $Mvk^{+/-}$ CD4 T cell growth in two MTT proliferation assays was heightened on average 15-fold as compared to that of $Mvk^{+/+}$ at 72 hours (data not shown). This may have been due to increased expression of CD28 on these cells.

In addition, we performed MTT proliferation assays in age- and sex-matched whole splenocyte cultures. At all time-points, $Mvk^{+/-}$ female splenocytes proliferated significantly less than those of $Mvk^{+/+}$ (24 hours, p=0.0046; 48 hours, p=0.0009; 72 hours, p<0.0001; analyzed by two-tailed Student's *t* test). The photograph in Fig. 6 illustrates an interesting pattern of proliferation in female $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocytes stimulated with Con-A at 48 hours. Note that in $Mvk^{+/-}$ there are more foci of proliferation than that of $Mvk^{+/+}$ but the clones do not expand to as high a density as those of $Mvk^{+/+}$.

Discussion

In these studies, we aimed to establish the activation status of splenic lymphocytes and macrophages in the gene-ablated mevalonate kinase-deficient mouse model. The $Mvk^{+/-}$ mouse model is a nearly faithful phenocopy of HIDS with elevated serum levels of TNFα, IgA and IgD, temperature dysregulation, splenomegaly and/or hepatomegaly, and hematological abnormalities including lymphocytosis, increased macrophage counts in peripheral blood, anemia, and abnormal platelet precursors. Because HIDS patients have periodic autoinflammatory crises, we postulated that $Mvk^{+/-}$mice may also undergo periodic or chronic inflammatory processes. If this is the case, inflammatory effects may be reflected in the status of basal levels of lymphocyte activation markers and also in the ability of an in vitro stimulus such as Con-A to induce activation of mixed splenocyte populations. The

studies presented here describe quantitative alterations in expression of activation markers in *Mvk*$^{+/-}$ splenocyte subpopulations at basal levels and in Con-A stimulated cultured cells as compared to *Mvk*$^{+/+}$ cells. The mechanism(s) by which these alterations occur have yet to be elucidated.

B7 glycoproteins are essential for initial T cell activation and regulation of the intensity of the ensuing immune response to antigenic stimuli. The B7 pathway provides a costimulatory signal to the initial signal transduced through the TCR-antigen/MHC complex. Balance between stimulatory and inhibitory signals is required for effective immune responses to pathogens and for maintaining self-tolerance. Without the B7 costimulatory signal, antigen-stimulated T cells become anergic (Chen and Nabavi 1994; Hara et al. 2006). Several disease states have been described where B7 glycoproteins CD80 and CD86 are abnormally expressed on the cell surface of APCs and also as soluble molecules that are able to bind T cell-associated B7 receptors and mediate the outcome of the disease state (Wong et al. 2005; Ip et al. 2006).

CD152 regulates T cell tolerance and autoimmunity (Fife and Bluestone 2008). Polymorphisms in the B7 loci have been linked to autoimmune susceptibility in humans and mice (Keir and Sharpe 2005). In two mouse models of autoimmunity, experimental autoimmune encephalomyelitis (EAE) and non-obese diabetic (NOD) autoimmune diabetes, B7 molecules have been shown to regulate peripheral T cell tolerance. Certain CD152 splice variants correlate with increased susceptibility to autoimmune diabetes in NOD mice and in Grave's disease, autoimmune hypothyroidism, and type I diabetes in humans. Aberrant expression of B7 family members are associated with other tissue-specific autoimmunity that develop in certain mouse strains as they age ranging between 5–14 months (Fife and Bluestone 2008). In our experiments, we used mice that were aged approximately 30 weeks.

B7 family protein structure, function, and expression depend on at least three products of the mevalonate pathway: 1) *dolichol* which mediates protein glycosylation; 2) *cholesterol* which is the main component of membrane lipid rafts; and 3) *geranylgeranyl-diphosphate* (GGDP) which is involved in protein prenylation. CD80 is one of the most glycosylated proteins on leukocyte surfaces (Davis et al. 2001). It has been suggested that changes in glycosylation of CD80 or other activation markers may greatly impact the outcome of an immune response (Greenfield et al. 1997). For example, altered glycoslyation patterns can lead to systemic autoimmune disease (Chui et al. 2001). Human IgA nephropathy has been linked to defective *O*-glycosylation of IgA1 (Baharaki et al. 1996; Hiki et al. 1999).

B7 glycoproteins, like many other membrane proteins, are prenylated by farnesyl-diphosphate or GGDP. Due to pleiotropic effects, and the complexity of regulation of the mevalonate pathway, it is not surprising that many experimental findings involving this pathway are paradoxical. This is reminiscent of our experimental system, in which the differences in expression level, either up or down, of B7 and other activation molecules were often approximately two-fold, sometimes having statistical significance, sometimes not. Nevertheless, it was striking that two-fold changes were so predominant. This phenomenon implies that a dose-dependent effect may be operative in our experiments. In this case, it may be a mevalonate kinase gene dosage effect in *Mvk* heterozygotes, directly or indirectly, impacting expression levels of several leukocyte activation markers.

It is possible that the aberrant expression of B7 glycoproteins we observed in *Mvk*$^{+/-}$ mice may be due to an aging effect on development of autoimmunity although aged-matched wildtype littermates were not affected in the same way. Perhaps *Mvk* mice carry an additional mutation such as one in the B7 or MHC loci that may predispose them for an "autoimmune" phenotype, however, one would expect wildtype littermates to exhibit similar

trends unless a mevalonate kinase deficiency exacerbates the phenotype. We believe the likely explanation for B7 expression aberration is due to defective post-translational modification, i.e., deficient prenylation and/or glycosylation which affects surface expression and may or may not play a role in establishment of autoimmunity in the HIDS mouse model. The results of our studies suggest the hypothesis that $Mvk^{+/-}$ mice undergo chronic immune stimulation from the environment, which in this disease model would deplete mevalonate products that B7 glycoproteins are dependent on for normal cell surface expression and function.

We also examined splenic $Mvk^{+/-}$ lymphocyte proliferation since the mevalonate pathway is critical to cell growth and its regulation (Cuthbert and Lipsky 1990; Mantha et al. 2005). We found that $Mvk^{+/-}$ male T cells and splenocytes had increased kinetics and levels of proliferation for up to 72 hours in culture. On the other hand, cultured $Mvk^{+/-}$ female splenocytes had significantly lower levels of proliferation than $Mvk^{+/+}$ females at all time-points. Supporting our observations are reports of gender effects during the use of statins in humans (Allen and Canadian Academic Detailing Corporation 2006; Ferrario 2008), although there have been no reports of gender effects in mevalonate kinase-deficient patients such as the association between gender and creatine kinase activity observed with statin consumption (Chan et al. 2006). We propose that the differences in proliferation between $Mvk^{+/+}$ and $Mvk^{+/-}$ females can be explained by the overexpression of CD152 on T cells and CD80 on APCs in $Mvk^{+/-}$ splenocytes. Increased expression of both CD152 and CD80 likely downregulated $Mvk^{+/-}$ female T cell proliferation through ligation of CD152 and CD80 evoking the B7 inhibitory pathway, especially in light of a significantly increased presence of $CD4^{+}Foxp3^{+}$ Tregs in $Mvk^{+/-}$ female spleens (unpublished observation). Consistent with this observation, human T cell proliferation is inhibited through CD80-CD152 interaction and is dependent on Tregs (Manzotti et al. 2002).

In conclusion, we report here that there are significant alterations in expression of B7 costimulatory molecules and other markers of activation in $Mvk^{+/-}$ splenocyte subpopulations. The aberrant expression of CD80 and CD152 support the classification of HIDS as an immunodeficiency disorder since the prevailing B7 interaction may have an inhibitory effect on the initiation of the T cell response to specific antigen. The data presented here provide the foundation for mechanistic studies to explain some of the immunological defects in HIDS patients and also provide possible cellular biomarker candidates of human HIDS that can be readily monitored for treatment efficacy in future clinical trials.

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References

Allen M. Canadian Academic Detailing Corporation. Statins in primary prevention: uncertainty in women, elderly. Am Fam Physician. 2006; 73:973–974. [PubMed: 16570733]

Baharaki D, Dueymes M, Perrichot R, Basset C, Le Corre R, Cledes J, Youinou P. Aberrant glycosylation of IgA from patients with IgA nephropathy. Glycoconj J. 1996; 13:505–511. [PubMed: 8872105]

Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Sharpe AH, Vallera DA. Opposing roles of CD28:B7 and CTLA-4:B7 pathways in regulating in vivo alloresponses in murine recipients of MHC disparate T cells. J Immunol. 1999; 162:6368–6377. [PubMed: 10352249]

Butscher WG, Powers C, Olive M, Vinson C, Gardner K. Coordinate transactivation of the interleukin-2 CD28 response element by c-Rel and ATF-1/CREB2. J Biol Chem. 1998; 273:552–560. [PubMed: 9417115]

Chen C, Nabavi N. In vitro induction of T cell anergy by blocking B7 and early T cell costimulatory molecule ETC-1/B7-2. Immunity. 1994; 1:147–154. [PubMed: 7534198]

Chui D, Sellakumar G, Green R, Sutton-Smith M, McQuistan T, Marek K, Morris H, Dell A, Marth J. Genetic remodeling of protein glycosylation in vivo induces autoimmune disease. Proc Natl Acad Sci USA. 2001; 98:1142–1147. [PubMed: 11158608]

Cuthbert JA, Lipsky PE. Inhibition by 6-fluoromevalonate demonstrates that mevalonate or one of the mevalonate phosphates is necessary for lymphocyte proliferation. J Biol Chem. 1990; 265:18568–18575. [PubMed: 2211719]

Davis SJ, Ikemizu S, Collins AV, Fennelly JA, Harlos K, Jones EY, Stuart DI. Crystallization and functional analysis of a soluble deglycosylated form of the human costimulatory molecule B7-1. Acta Crystallogr D Biol Crystallogr. 2001; 57:605–608. [PubMed: 11264596]

Ferrario CM. Of gender, statins, and coronary artery interventions. Ther Adv Cardiovasc Dis. 2008; 2:73–74. [PubMed: 19124409]

Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev. 2008; 224:166–182. [PubMed: 18759926]

Freeman GJ, Borriello F, Hodes RJ, Reiser H, Gribben JJ, Ng JW, Kim J, Goldberg JM, Hathcock KGL, Lombard LA, Wang S, Gray G, Nadler LM, Sharpe AH. Murine B7-2, and alternative CTLA-4 counter-receptor that costimulates T cell proliferation and interleukin-2 production. J Exp Med. 1993; 178:2185–2192. [PubMed: 7504059]

Goldfinger S. The inherited autoinflammatory syndrome: a decade of discovery. Trans Am Clin Climatol Assoc. 2009; 120:413–418. [PubMed: 19768193]

Greenfield EA, Howard E, Paradis T, Nguyen K, Benazzo F, McLean P, Höllsberg P, Davis G, Hafler DA, Sharpe AH, Freeman GJ, Kuchroo VK. B7.2 expressed by T cells does not induce CD28-mediated costimulatory activity but retains CTLA4 binding: implications for induction of antitumor immunity to T cell tumors. J Immunol. 1997; 158:2025–2034. [PubMed: 9036945]

Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005; 23:515–548. [PubMed: 15771580]

Hager EJ, Tse HM, Piganelli JD, Gupta M, Baetscher M, Tse TE, Pappu AS, Steiner RD, Hoffmann GF, Gibson KM. Deletion of a single mevalonate kinase (Mvk) allele yields a murine model of hyper-IgD syndrome. J Inherit Metab Dis. 2007; 30:888–895. [PubMed: 18008182]

Hara Y, Kitazawa Y, Funeshima N, Kawasaki M, Sato Y, Tezuka K, Kimura H, Hatakeyama K, Li XK. Anergic lymphocytes generated by blocking CD28 and ICOS pathways in vitro prolong rat cardiac graft survival. Int Immunopharmacol. 2006; 6:1143–1151. [PubMed: 16714218]

Hiki Y, Kokubo T, Iwase H, Masaki Y, Sano T, Tanaka A, Toma K, Hotta K, Kobayashi Y. Underglycosylation of IgA1 hinge plays a certain role for its glomerular deposition in IgA nephropathy. J Am Soc Nephrol. 1999; 10:760–769. [PubMed: 10203360]

Ip WK, Wong CK, Leung TF, Lam CW. Plasma concentrations of soluble CTLA-4, CD28, CD80 and CD86 costimulatory molecules reflect disease severity of acute asthma in children. Pediatr Pulmonol. 2006; 41:674–682. [PubMed: 16703581]

Jenkins MK, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol. 1991; 147:2461–2466. [PubMed: 1717561]

Kearney ER, Pape KA, Loh DY, Jenkins MK. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity. 1994; 1:327–339. [PubMed: 7889419]

Keir ME, Sharpe AH. The B7/CD28 costimulatory family in autoimmunity. Immunol Rev. 2005; 204:128–143. [PubMed: 15790355]

Kim YC, Kim KK, Shevach EM. Simvastatin induces Foxp3+ T regulatory cells by modulation of transforming growth factor-beta signal transduction. Immunology. 2010; 130(4):489–493.

Krummel MF, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J Exp Med. 1996; 183:2533–2540. [PubMed: 8676074]

Li R, Perez N, Karumuthil-Melethil S, Prabhakar BS, Holterman MJ, Vasu C. Enhanced engagement of CTLA-4 induces antigen-specific CD4+CD25+Foxp3+ and CD4+CD25 TGF-beta1+ adaptive regulatory T cells. J Immunol. 2007; 179:5191–5203. [PubMed: 17911604]

Lohr J, Knoechel B, Jiang S, Sharpe AH, Abbas AK. The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens. Nat Immunol. 2003; 4:664–669. [PubMed: 12766766]

Mantha AJ, Hanson JE, Goss G, Lagarde AE, Lorimer IA, Dimitroulakos J. Targeting the mevalonate pathway inhibits the function of the epidermal growth factor receptor. Clin Cancer Res. 2005; 11:2398–2407. [PubMed: 15788691]

Manzotti CN, Tipping H, Perry LC, Mead KI, Blair PJ, Zheng Y, Sansom DM. Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells. Eur J Immunol. 2002; 32:2888–2896. [PubMed: 12355442]

McAdam AJ, Chang TT, Lumelsky AE, Greenfield EA, Boussiotis VA, Duke-Cohan JS, Chernova T, Malenkovich N, Jabs C, Kuchroo VK, Ling V, Collins M, Sharpe AH, Freeman GJ. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. J Immunol. 2000; 165:5035–5040. [PubMed: 11046032]

Notarangelo LD, Fischer A, Geha RS, Casanova JL, Chapel H, Conley ME, Cunningham-Rundles C, Etzioni A, Hammarström L, Nonoyama S, Ochs HD, Puck J, Roifman C, Seger R, Wedgwood J. Primary immunodeficiencies: 2009 update. J Allergy Clin Immunol. 2009; 124:1161–1178. [PubMed: 20004777]

Perez N, Karumuthil-Melethil S, Li R, Prabhakar BS, Holterman MJ, Vasu C. Preferential costimulation by CD80 results in IL-10-dependent TGF-beta1(+) -adaptive regulatory T cell generation. J Immunol. 2008; 180:6566–6576. [PubMed: 18453575]

Perkins D, Wang Z, Donovan C, He H, Mark D, Guan G, Wang Y, Walunas T, Bluestone J, Listman J, Finn PW. Regulation of CTLA4 expression during T cell activation. J Immunol. 1996; 32:366–373.

Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol. 2001; 19:225–252. [PubMed: 11244036]

Sethna MP, van Parijs L, Sharpe AH, Abbas AK, Freeman GJ. A negative regulatory function of B7 revealed in B7-1 transgenic mice. Immunity. 1994; 1:415–421. [PubMed: 7533646]

Taylor PA, Lees CJ, Fournier S, Allison JP, Sharpe AH, Blazar BR. B7 expression on T cells down-regulates immune responses through CTLA-4 ligation via T-T interactions. J Immunol. 2004; 172:34–39. [PubMed: 14688306]

Vasu C, Prabhakar BS, Holterman MJ. Targeted CTLA-4 engagement induces $CD4^{+}CD25^{+}CTLA\text{-}4^{high}$ T regulatory cells with target (allo) antigen specificity. J Immunol. 2004; 173:2866–2876. [PubMed: 15295006]

Wentworth JM, Naselli G, Brown WA, et al. Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. Diabetes. 2010; 59(7):1648–1656. [PubMed: 20357360]

Wong CK, Lit LC, Tam LS, Li EK, Lam CW. Aberrant production of soluble costimulatory molecules CTLA-4, CD28, CD80 and CD86 in patients with systemic lupus erythematosus. Rheumatology. 2005; 44:989–994. [PubMed: 15870153]

Abbreviations

APC	Antigen presenting cell
CFSE	5-6-carboxyfluorescein diacetate, succinimidyl ester

Con-A	Concanavalin-A
GGDP	Geranylgeranyl-diphosphate
EAE	Experimental autoimmune encephalomyelitis
Foxp3	Forkhead box P3 transcription factor
HIDS	Human hyper-IgD syndrome
IL-	Interleukin-
MCP-1	Macrophage chemoattractant protein 1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility antigen
MVK	Mevalonate kinase
NOD	Non-obese diabetic
TCR	T cell antigen receptor
TH	T helper cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell

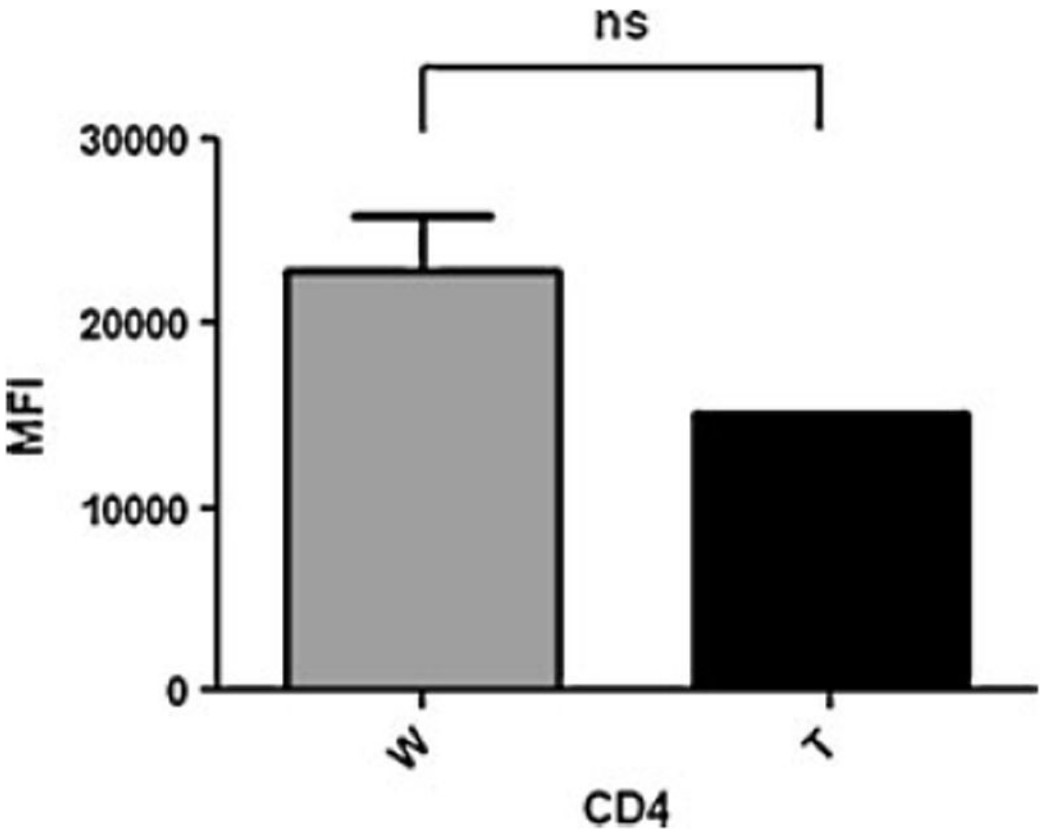

Fig. 1.

Basal CD4 expression in CD3ε^{+} T cells of *Mvk*$^{+/+}$ (wild-type, W) and *Mvk*$^{+/-}$ (heterozygote, T) splenocyte populations. The differences in the means (± SEM) of MFI in two experiments were not significant (ns), $p>0.05$. Data were analyzed by one-tailed Student's *t* test

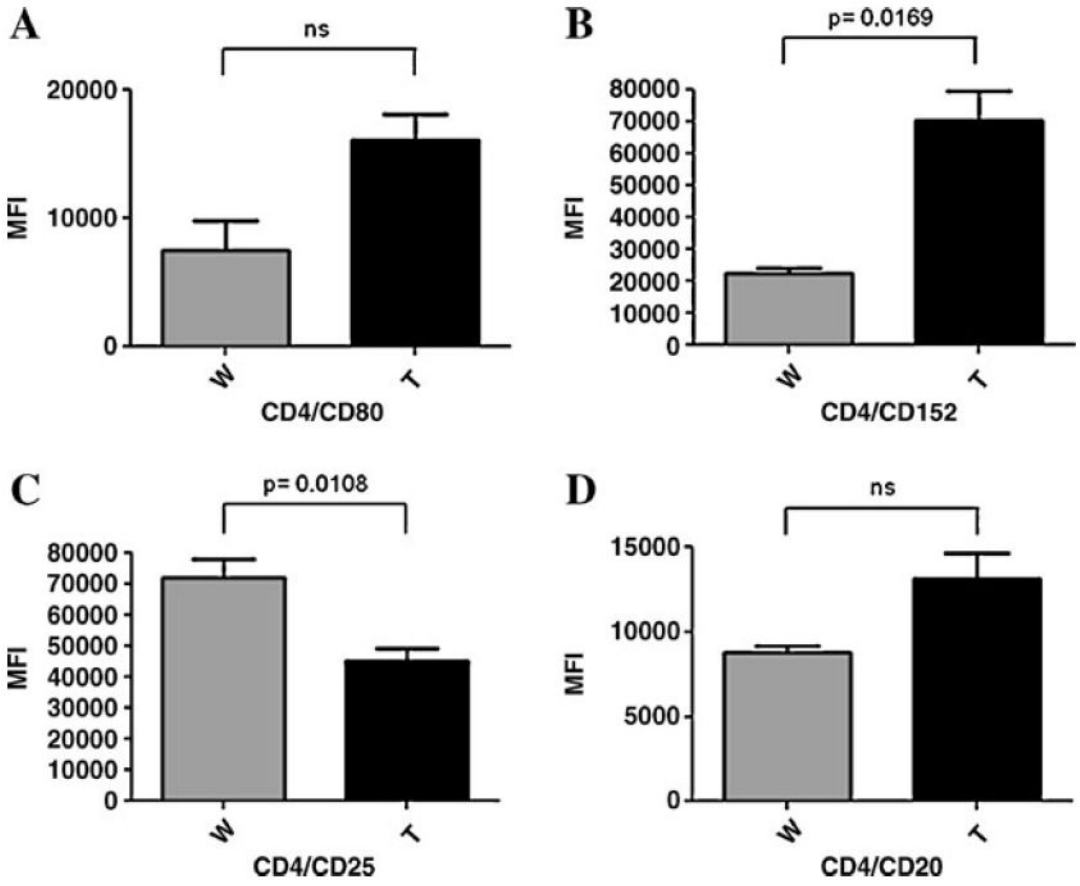

Fig. 2.

(**a**) Basal CD80 expression in CD4 T cells of $Mvk^{+/+}$ (wild-type, W) and $Mvk^{+/-}$ (heterozygote, T) splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was not significant (ns), p>0.05. (**b**) Basal CD152 expression in CD4 T cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was significant, p=0.0169. (c) Con-A stimulated CD25 expression in CD4 T cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was significant, p=0.0108. (**d**) Con-A stimulated CD28 expression in CD4 T cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was not significant, p > 0.05. Data were analyzed by one-tailed Student's *t* test

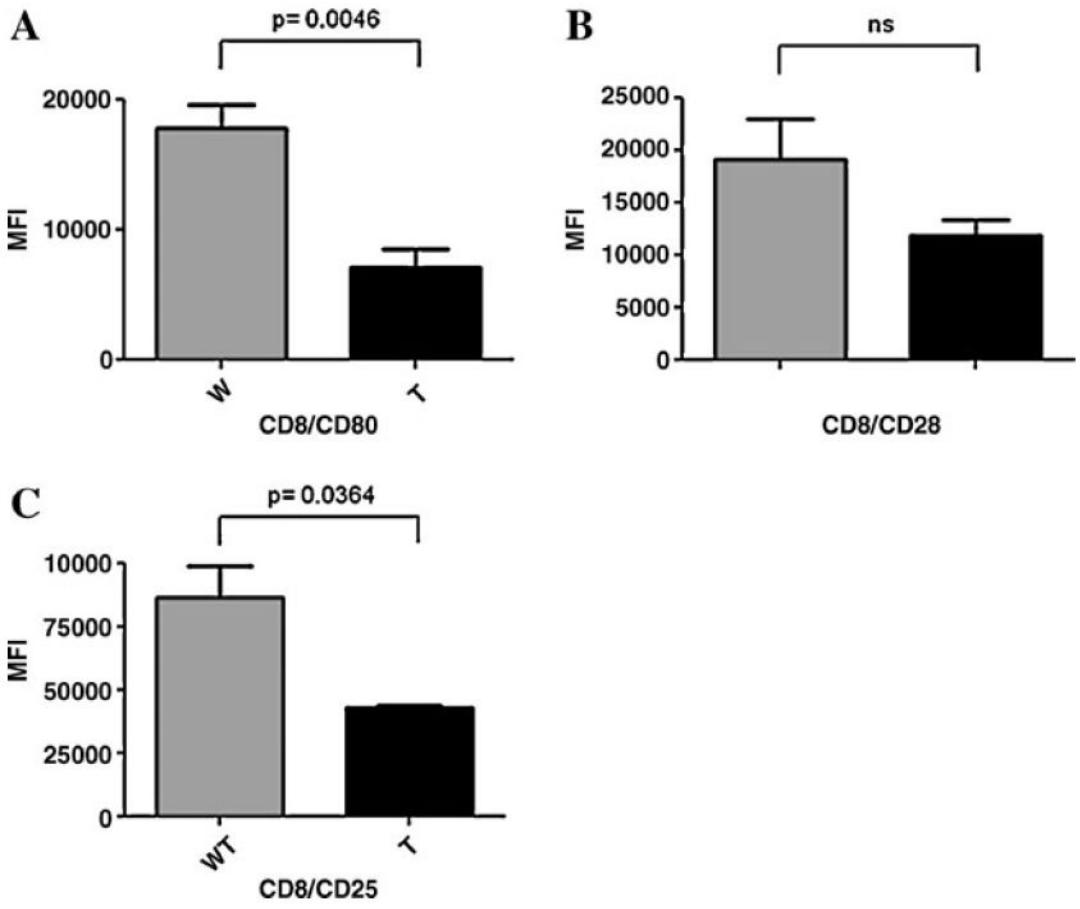

Fig. 3.

(**a**) Basal CD80 expression in CD8 T cells of $Mvk^{+/+}$ (wild-type, W) and $Mvk^{+/-}$ (heterozygote, T) splenocyte populations. The difference in the means (± SEM) of MFI in three experiments was significant, p=0.0046. (**b**) Basal CD28 expression in CD8 T cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was not significant (ns), p>0.05. (**c**) Con-A stimulated CD25 expression in CD8 T cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in three experiments was significant, p=0.0364. Data were analyzed by one-tailed Student's *t* test

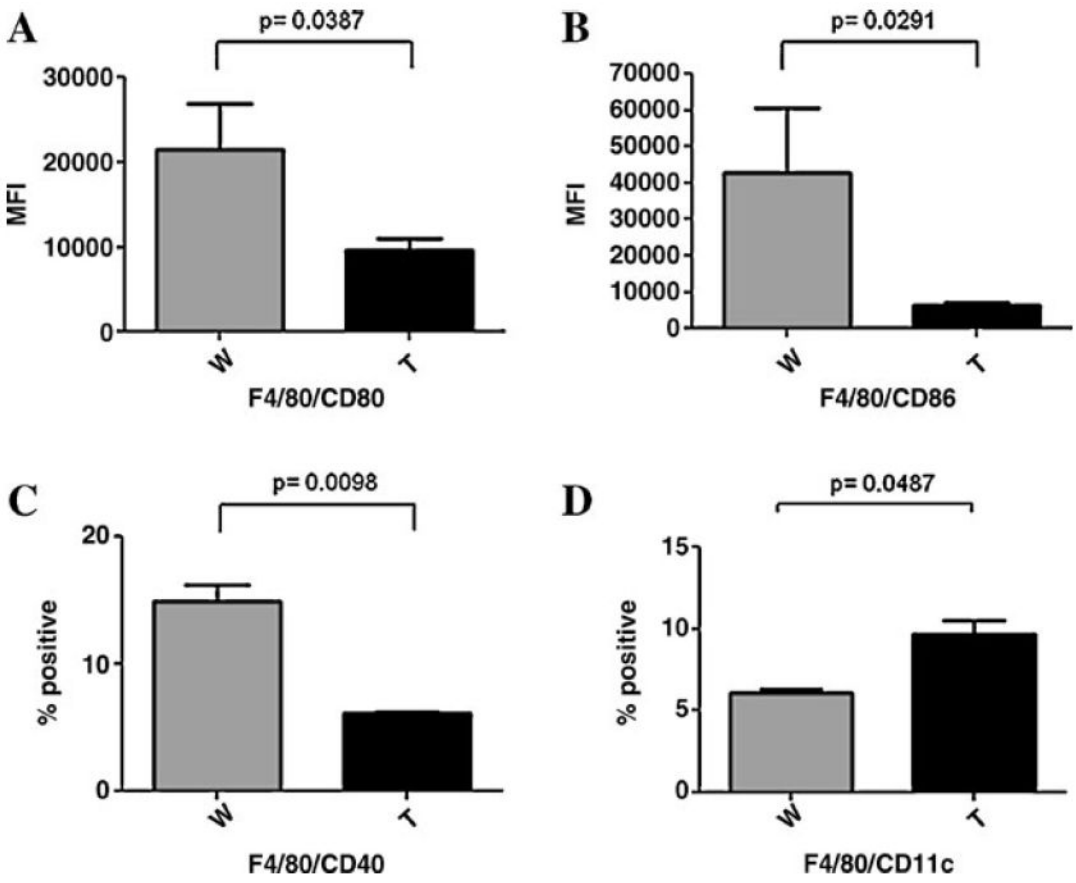

Fig. 4.

(**a**) Basal CD80 expression in F4/80^{+} macrophages of *Mvk*$^{+/+}$ (wild-type, W) and *Mvk*$^{+/-}$ (heterozygote, T) splenocyte populations. The difference in the means (± SEM) of MFI in three experiments was significant, p=0.0387. (**b**) Basal CD86 expression in F4/80^{+} macrophages of *Mvk*$^{+/+}$ and *Mvk*$^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in three experiments was significant, p=0.0291. (**c**) Basal CD40 % positive of F4/80^{+} macrophages in *Mvk*$^{+/+}$ and *Mvk*$^{+/-}$ splenocyte populations. The difference in the means (± SEM) of % positive in two experiments was significant, p=0.0098. (**d**) Basal CD11c % positive of F4/80^{+} macrophages in *Mvk*$^{+/+}$ and *Mvk*$^{+/-}$ splenocyte populations. The difference in the means (± SEM) of % positive in two experiments was significant, p=0.0487. Data were analyzed by one-tailed Student's *t* test except for CD11c which was analayzed by two-tailed Student's *t* test

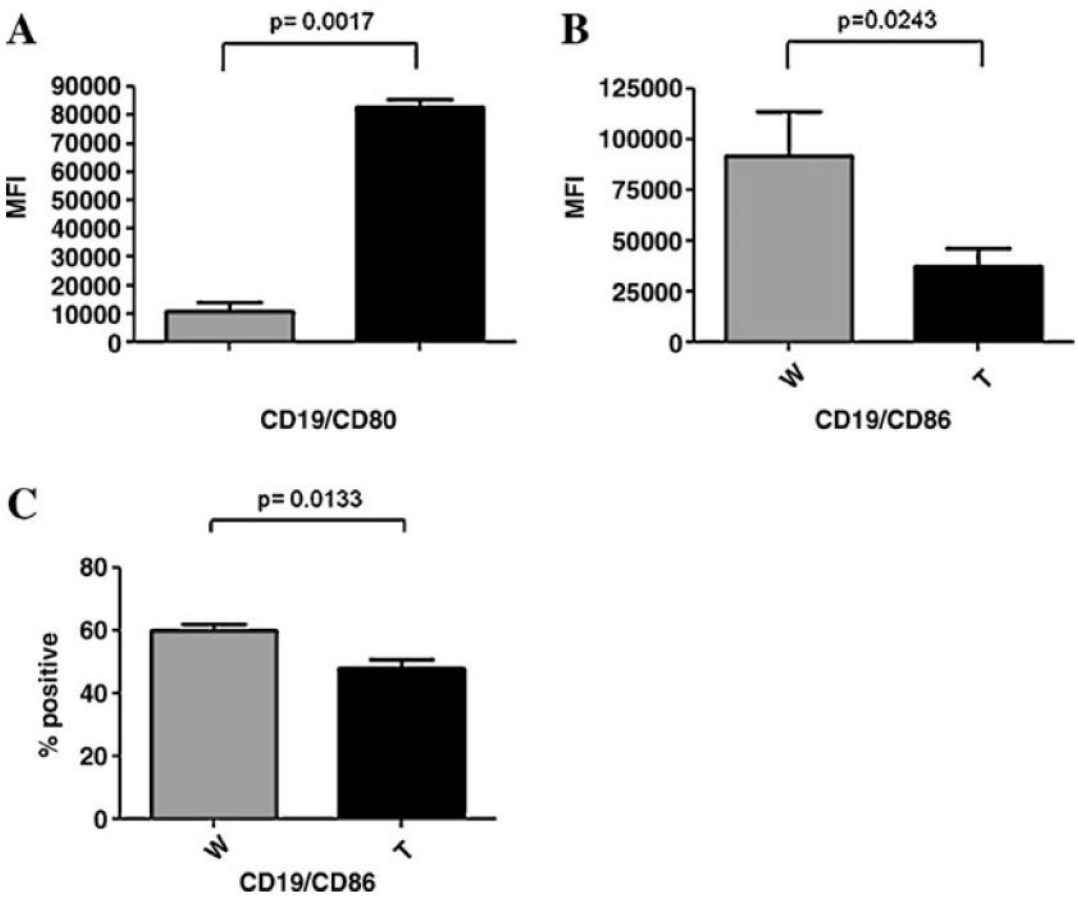

Fig. 5.

(**a**) Con-A stimulated CD80 expression in $CD19^+$ B cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was significant, p=0.0017. (**b**) Con-A stimulated CD86 expression in $CD19^+$ B cells of $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of MFI in two experiments was significant, p=0.0243. (**c**) Con-A stimulated CD86 % positive of $F4/80^+$ macrophages in $Mvk^{+/+}$ and $Mvk^{+/-}$ splenocyte populations. The difference in the means (± SEM) of % positive in two experiments was significant, p=0.0133. Data were analyzed by one-tailed Student's *t* test

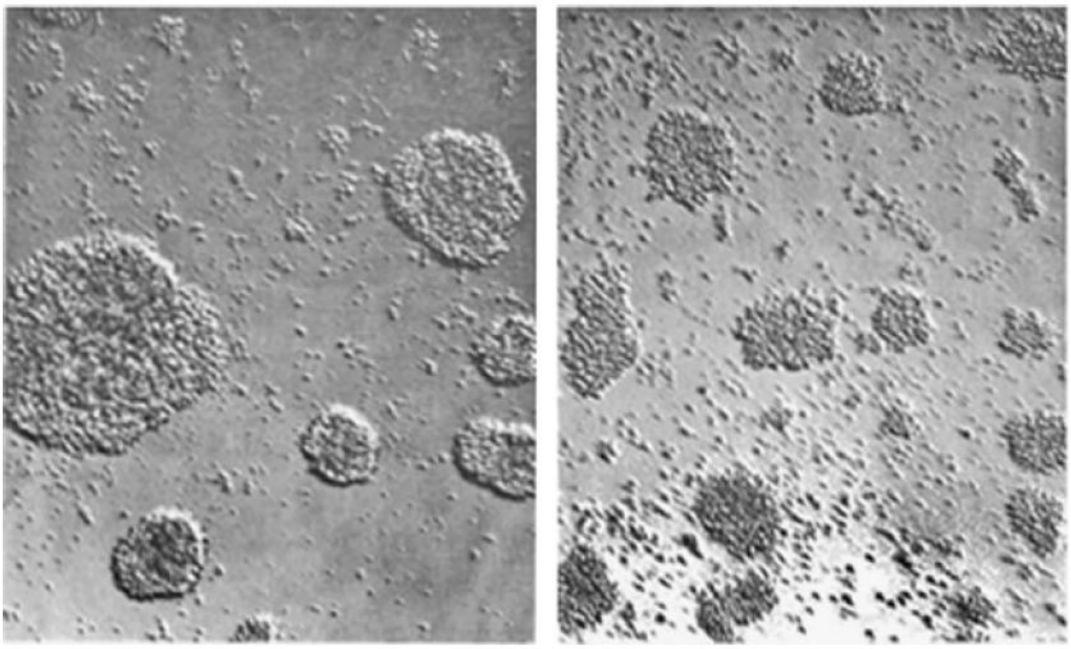

Fig. 6.
Photomicroscopic images of female *Mvk*$^{+/+}$ (left) and *Mvk*$^{+/-}$ (right) Con-A stimulated cultures after 48 hours of proliferation. Magnification 400x

Table 1

Cell markers evaluated in this study

B lymphocytes	T lymphocytes	Macrophages
IgD	CD3	F4/80
IgM	CD4	CD16/32
IgG	CD8	CD40
CD19	CD25	CD80
CD45R	CD28	CD86
CD25	CD152	CD11a
CD11a	CD154	CD11b
CD11b	CD80	CD11c
CD11c	CD86	CD62L
CD23	ICOS	MHC I
CD62L	CD62L	MHC II
CD69	CD69	
CD80	CD11a	
CD86	CD44	
CD40	MHC I	
CD17/32		
MHC I		
MHC II		