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DAP12 Promotes IRAK-M Expression and IL-10 Production by Liver Myeloid Dendritic Cells and Restrains Their T Cell Allostimulatory Ability

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Freshly isolated hepatic dendritic cells (DC) are comparatively immature, relatively resistant to maturation, and can downmodulate effector T cell responses. Molecular mechanisms that underlie these properties are ill defined. DNAX-activating protein of 12 kDa (DAP12) is an ITAM-bearing transmembrane adaptor protein that integrates signals through several receptors, including triggering receptor expressed on myeloid cells-1, -2, and CD200R. Notably, DC propagated from DAP12-deficient mice exhibit enhanced maturation in response to TLR ligation. Given the constitutive exposure of liver DC to endotoxin draining from the gut, we hypothesized that DAP12 might regulate liver DC maturation. We show that DAP12 is expressed by freshly isolated liver, spleen, kidney, and lung myeloid DC. Moreover, inhibition of DAP12 expression by liver DC using small interfering RNA promotes their phenotypic and functional maturation, resulting in enhanced TNF-α, IL-6, and IL-12p70 production, reduced secretion of IL-10, and enhanced CD4+ and CD8+ T cell proliferation. Furthermore, DAP12 silencing correlates with decreased STAT3 phosphorylation in mature liver DC and with diminished expression of the IL-1R–associated kinase-M, a negative regulator of TLR signaling. These findings highlight a regulatory role for DAP12 in hepatic DC maturation, and suggest a mechanism whereby this function may be induced/maintained. The Journal of Immunology, 2011, 186: 1970–1980.

The liver is a site of both immune regulation and tolerance induction (1–5). Portal venous Ag administration is tolerogenic (6, 7), and oral tolerance requires the liver (8). Also, liver grafts between MHC-mismatched mouse (9) and certain rat strains (10) are accepted without immunosuppressive therapy and can promote donor-specific tolerance (9, 11). These properties may reflect the unusual complement of immune cells within the liver microenvironment and their functions and inter-actions. Thus, multiple hematopoietic and nonhematopoietic APC populations with the potential to regulate immunity reside in the liver, including dendritic cell (DC) subsets (12–15), Kupffer cells (16), stellate cells (17), and sinusoid-lining endothelial cells (18). In particular, DC are well-recognized to mediate the balance between immune activation and regulation (19–21).

The liver microenvironment drives the differentiation of IL-10–secreting (22) interstitial immature DC (12, 23), which can downmodulate T cell responses (24, 25) and promote tolerance in models of auto-(23, 26, 27) and allografting (24, 28). Moreover, constitutive ablation of CD11c+ DC in mice increases IL-17 production by hepatic CD4+ T cells (29). Due to the location of the liver downstream from the gut, hepatic DC are exposed continually to commensal microbial products that appear to induce hypersensitiveness to LPS (endotoxin tolerance) (30, 31) and other TLR ligands (32) in these cells. At the molecular level, bacterial products stimulate negative regulation of TLR signaling via IL-6–driven STAT3 activation (33, 34) and IL-1R–associated kinase-M (IRAK-M) expression (34) that may underlie the refractory state of liver compared with blood-borne or lymphoid tissue DC (30).

DNAX-activating protein of 12 kDa (DAP12) is a homodimeric ITAM-bearing transmembrane adaptor protein. It is highly expressed in lymphoid tissues, in the lung, and to a lesser extent in the liver (35). Within these tissues, DAP12 is expressed in NK cells, macrophages, and DC. DAP12 integrates signals through multiple receptors, including triggering receptor expressed on myeloid cells (TREM)-1 and -2, NKG2D, Ly49, myeloid DAP12-associating lectin-1, and CD200R (36–38). By associating with distinct receptors, DAP12 can potentiate or inhibit leukocyte activation, with the outcome determined by the avidity of the interaction between the DAP12-associated receptor and its ligand (39). This is consistent with the divergent immunological outcomes observed in mice globally deficient in DAP12. DAP12−/−...
mice exhibited enhanced TLR responses (40), and the absence of DAP12 accelerates disease development in NOD mice (41). In contrast, DAP12−/− mice have exhibited resistance to endotoxemia and septic shock (42) and the induction of experimental allergic encephalomyelitis (43). Thus, there is a lack of clear understanding of the precise mechanistic and functional role that DAP12 plays in regulating immune responses under quiescent and pathological conditions.

It has been reported that, with or without LPS stimulation, conventional myeloid DC (mDC) propagated from the bone marrow (BM [BM-DC]) of DAP12−/− mice are more mature, displaying enhanced costimulatory molecule expression (44, 45). Furthermore, following CMV infection or CpG stimulation, splenic plasmacytoid DC from DAP12-deficient mice exhibited augmented IL-12 production (46). Few studies have evaluated the role of DAP12 in regulation of peripheral DC function. There is evidence, however, that pulmonary CD11c+ APC from DAP12-deficient mice have increased NF-κB activation, enhanced cytokine responses to TLR ligation, and induced augmented Th1 responses following local microbial infection (47). Moreover, adoptive transfer of DAP12-deficient pulmonary CD11c+ APC enhances Ag-specific T cell responses in vivo (47). These findings suggest that DAP12 may regulate DC maturation and function in nonlymphoid tissue.

In this study, we have examined for the first time the function of DAP12 in primary mouse mDC, especially those isolated from the liver. We show that these liver DC constitutively express DAP12 and that reducing DAP12 expression in liver DC enhances their phenotypic and functional maturation. Reduction of DAP12 in liver DC increases secretion of TNF-α, IL-6, and IL-12p70, but impairs IL-10 production while increasing T cell stimulatory capacity. Significantly, diminished DAP12 expression correlates with decreased STAT3 phosphorylation in mature DC in the absence of exogenous stimuli. Likewise, diminished DAP12 expression in liver DC corresponds with reduced IRAK-M expression and enhanced responses to LPS. To our knowledge, these data highlight for the first time a regulatory role for DAP12 in hepatic DC.

Materials and Methods

Mouse

Male C57BL/6 (H2b), C57BL/10 (H2a), and BALB/c (H2d) mice, aged 8–12 wk, were purchased from The Jackson Laboratory. DAP12−/− mice (48), backcrossed onto the C57BL/6 background, were bred from pairs kindly provided by Dr. Marco Colonna, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO. Mice were treated for 10 d with the endogenous DC poietin Flt3 ligand (Chinese hamster ovary cell-derived recombinant human Flt3 ligand; Angen; 10 μg/dg [49]) prior to cell isolation. All mice were housed in the specific pathogen-free central animal facility of the University of Pittsburgh School of Medicine with access to food and water ad libitum. Experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol and in accordance with National Institutes of Health guidelines.

Isolation of mDC and transfection with DAP12 small inhibitory RNA

Liver (14), kidney (50), and lung (51) nonparenchymal cells were isolated as described. For mDC purification, RBC-depleted nonparenchymal cells or pneumocytes were incubated first with biotin-conjugated mAb against NK1.1 and streptavidin microbeads (Miltenyi Biotec), then with anti-plasmacytoid DC Ag (PDCA-1)-conjugated microbeads. The NK1.1−PDCA1+ cells were then incubated with anti-CD11c microbeads. The purity of CD11c−NK1.1−PDCA1+ cells (hereafter referred to as DC) was routinely >97% for liver DC, >92% for kidney DC, and >90% for lung DC. The DC were transfected overnight with either DAP12 small inhibitory RNA (siRNA; 400 nM) or negative (−) control (i) siRNA (Ambion) using GeneSilencer (Genlantis) in accordance with the manufacturer’s protocol. In some experiments, DC were transfected, stimulated 2 h later with LPS, then incubated overnight.

Flow cytometry and CFSE-dilution assays

The following directly conjugated rat anti-mouse Abs were used for staining of freshly isolated DC: CD11c-Pacific Blue, B220–PE-Cy5, TREM-1–PE, or TREM-2–PE (R&D Systems) or CD200R–PE (Biolegend). In some experiments, DC were transfected overnight (18 h) with DAPI or cili siRNA, then labeled with CD11c-Pacific Blue, B220–PE-Cy5, Ly6-FITC, CD80–allophycocyanin, and CD86–PE or with CD11c-Pacific Blue, B220–PE-Cy5, and B7-H1–PE, B7-H2–PE, B7-H3–PE, B7-H4–PE, B7-RI–PE, or CR7–PE. In other experiments, 2 h following transfection, the DC were incubated overnight with recombinant mouse IL-6 or IL-10 (1 ng/ml; PeproTech), then labeled with CD11c-Pacific Blue, B220–PE-Cy5, h-Ig–FITC, CD80–allophycocyanin, and CD86–PE. A minimum of 20,000 events in the fluorescence positive gate was acquired using a LSRII flow cytometer (BD Biosciences). For proliferation analysis, T cells (BALB/c) were first purified by negative selection from RBC-depleted splenocyte suspensions, incubated in the absence of LPS overnight with recombinant mouse IL-6 or IL-10 (1 ng/ml; PeproTech), then labeled with CD11c-Pacific Blue, B220–PE-Cy5, h-Ig–FITC, CD80–allophycocyanin, and CD86–PE. A minimum of 20,000 events was acquired. Data were analyzed using FlowJo 7 (Tree Star).

RNA isolation and semiquantitative RT-PCR

Total RNA was extracted using TRizol (Invitrogen) and then reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad). DAPI12, IRAK-M, and β-actin DNA products were amplified with Fast SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7000 Fast Sequence Detection System (Applied Biosystems). Data were plotted using the manufacturer’s software as the ΔRn fluorescence signal versus cycle number. Cycle threshold number was determined as the cycle number at which the ΔRn crosses the threshold. Relative gene expression was determined by comparing to a standard curve and then normalized to expression of β-actin using the comparative cycle threshold method (52). Primers used for DAPI12 were forward: 5′-CGTACAGCCCAGAGTGAC-3′ and reverse: 5′-CACCC-AAGTCACCCAGAACAA-3′; for β-actin, forward: 5′-AGGAGGAAATC-GTGCCTGAC-3′ and reverse: 5′-GAATCTGATGACCTGGCGGT-3′; and for IRAK-M, forward: 5′-TGACACAGGCAGGACCCTF-3′ and reverse: 5′-GATTCGAACGGTCCAGGAA-3′.

Western blot analysis for STAT3

DC transfected overnight with DAPI12 siRNA were incubated for 30 min with IL-6 or IL-10 (1 ng/ml). Cells were collected on ice, washed twice with ice-cold PBS, and then used for Western blot analysis. Total cell lysates (50 μg) were resolved on 10% gels, transferred to polyvinylidene difluoride membranes, and blocked with 5% milk in TBST. Membranes were probed with Abs specific for p-Y705, p-Y705–STAT3 was detected in cells transfected overnight, fixed with 1.5% paraformaldehyde, then permeabilized with 100% methanol. Nonspecific binding on permeabilized cells was blocked using anti-CD16/32 and 2% goat serum, then cells were transfected with CD11c-Pacific Blue, CD80-APC, and p-Y705–STAT3–PE (BD Biosciences) at room temperature. A minimum of 50,000 events was acquired. Data were analyzed using FlowJo 7 (Tree Star).

ELISA

ELISA kits for IL-4, IL-10, IL-12p70, IL-17, TNF-α, or IFN-γ were purchased from Biologic and IL-6 ELISA kits from eBioscience. Samples were assayed in triplicate in accordance with the manufacturer’s protocol.

Ex vivo T cell proliferative responses and cytokine production

Liber DC (2.5 × 105) isolated from either DAPI12−/− or control C57BL/6 mice were adoptively transferred via the lateral tail vein into allogeneic BALB/c recipients. At day 7, the recipient mice were euthanized and their spleens excised, RBCs lysed, and T cells isolated as described above. Bulk T cells (1 × 106) were then cultured with gamma-irradiated (2000 rad)
C57BL/6 splenocytes (2.5 × 10^6) for 72 h. Cocultures were pulsed with [3H]thymidine (1.0 μCi/well) for the last 18 h and proliferation measured as counts per minute using a Topcount NTX microplate scintillation and luminescence counter (PerkinElmer). Data were normalized to the proliferation of T cells (BALB/c) isolated from naïve mice and stimulated with C57BL/6 splenocytes in parallel. Some responder populations were restimulated for either 6 or 18 h with PMA (50 ng/ml) and ionomycin (2.5 μM) in the presence of GolgiPlug (BD Biosciences) and then stained with CD8–PE-Cy7, CD4-Pacific Blue (both from Biolegend), and IL-4–PE, together with IFN-γ–allophycocyanin or IL-17–PE or IL-10–PE (all from BD Biosciences) (18 h stimulation). In supplemental experiments, similar staining was performed on T cells isolated from BALB/c recipients of C57BL/6 liver DC that had been transfected with either (−) ctl or DAP12 siRNA, as described above.

**Statistical analyses**

Statistical analyses were performed with either Student t tests or two-way ANOVA, with Bonferroni post hoc comparisons when appropriate, using Prism v4 (GraphPad). A p value <0.05 was deemed significant.

**Results**

**Liver DC express DAP12 mRNA that is upregulated following stimulation with physiological concentrations of LPS**

Although factors that regulate DAP12 expression in DC have yet to be elucidated, there is evidence that DAP12 mRNA expression in a murine myeloid cell line is increased in the presence of LPS (35). We hypothesized that DAP12 would be expressed in freshly isolated liver DC that are exposed constitutively to gut-derived LPS and other microbial products in vivo. Given the well-documented expression of DAP12 in NK cells (36), we evaluated DAP12 expression in highly purified, NK1.1-depleted liver and spleen CD11c^+ DC (Fig. 1A). Consistently, ∼2% of cells expressed NK1.1. Because these NK1.1^+ cells also expressed CD11c, we assumed that these very minor contaminants were NK-DC rather than NK cells. RT-PCR revealed that both freshly isolated C57BL/6 and C57BL/10 liver and spleen DC expressed DAP12 mRNA at similar or higher levels than NK cells purified from the same tissues (Fig. 1B). We were interested to know whether DAP12 was expressed similarly by DC from other mouse nonlymphoid tissues. Thus, we evaluated DAP12 expression in DC isolated from the lung and kidney relative to that expressed by liver DC. As shown in Fig. 1C, liver DC expressed higher levels of DAP12 than DC isolated from the other nonlymphoid tissues. Elsewhere, it has been reported (41) that DAP12 expression is diminished in splenic DC cultured in vitro. We observed that following overnight culture, liver DC maintained a higher level of DAP12 RNA expression than splenic DC (data not shown). We hypothesized that LPS, constantly draining into the liver, drives the expression of DAP12 in liver DC. Our data show that stimulation of liver DC with physiological concentrations of LPS (0.1 ng/ml) within the range reported for portal blood (100 pg/ml to 1 ng/ml) (53) caused an increase in DAP12 expression. Following stimulation with supraphysiological concentrations of LPS, however, DAP12 mRNA in liver DC was diminished (Fig. 1D). This latter finding contrasts with previous work using macrophage-related cell lines (35), possibly reflecting intrinsic differences in the regulation of DAP12 expression by different cell types.

**Reducing DAP12 expression increases costimulatory and coregulatory molecule expression on liver and splenic DC**

Liver DC are phenotypically less mature than splenic DC (5). We hypothesized that reducing the level of DAP12 in liver DC might enable mechanisms required for maturation to occur. DAP12^−/− mice have impaired Ag-presenting capacity in vivo (43, 44), which may reflect either a direct influence of DAP12 deficiency on DC function or the absence of DAP12 in other cell types. We adopted an siRNA-based strategy to evaluate more specifically the role of DAP12 in controlling the maturation status of liver DC. Liver DC transfected overnight with ctl siRNA maintained a significantly higher level of DAP12 expression relative to similarly treated splenic DC (Fig. 2A). Transfection with DAP12-specific siRNA reduced DAP12 expression 63 ± 4.0% in liver DC and 67 ± 25% in splenic DC (mean ± SD, n = 4; data shown are representative).

Transfection of BM-DC with siRNA does not alter expression of costimulatory molecules (54, 55). Likewise, we found that introducing siRNA into splenic DC had little effect on costimulatory molecule expression. However, transfection of liver DC with ctl siRNA increased CD80 and CD86 expression compared with nontransfected controls (data not shown). Reduction of DAP12 mRNA in both liver and splenic DC resulted in significantly increased expression of CD80 and CD86 as well as of the coregulatory molecules B7-H1 (programmed death ligand-1) and B7-H2 (programmed death ligand-2) relative to DC transfected with ctl siRNA (Fig. 2B, 2C). Reduction of DAP12 mRNA also increased CD80 and CD86 expression on a distinct population of liver but not spleen DC, reflected by an increase in the percentage of positive cells relative to the isotype control (Fig. 2B, 2D). Expression on either liver or spleen DC of other B7 family members that have been implicated in regulation of T cell activation (B7-H3, B7-H4, and B7-RP) (56), MHC class II, as well as the chemokine receptor CCR7, reported previously to be upreg-

**FIGURE 1.** Freshly isolated murine (C57BL/6) liver and spleen CD11c^+ myeloid DC express the membrane adaptor protein DAP12. A, Purity of NK1.1-depleted liver and spleen CD11c^+ DC. Data shown are gated on live cells by forward and side scatter. The mean percentage of NK1.1^+ cells is shown above the insets. B, DAP12 mRNA expression in CD11c^−NK1.1^−PDCA-1^− DC and in NK1.1^+ cells (positive control) purified from livers and spleens. C, DAP12 expression in C57BL/6 mouse lung or kidney DC compared with liver DC. D, DAP12 mRNA expression in liver DC stimulated with the indicated concentrations of LPS for 18 h. In B–D, expression of DAP12 was determined by semiquantitative RT-PCR. Data are representative of three independent experiments for all but C, in which DAP12 expression was evaluated in kidney DC in two independent experiments. Bars are means ± 1 SD of triplicate values. *p < 0.05, **p < 0.01. AU, arbitrary units.
ulated on human DC by DAP12 activation (57), was not altered significantly by transfection with DAP12 siRNA (Fig. 2C).

**Loss of DAP12 upregulates proinflammatory cytokine production and reduces IL-10 production by liver DC**

In keeping with a previously reported (45) role for DAP12 as a negative regulator of proinflammatory cytokine secretion by mouse BM-DC, loss of DAP12 resulted in increased secretion of the proinflammatory cytokines IL-6, IL-12p70, and TNF-α (Fig. 3). No change in IL-1β secretion was observed (data not shown). The secretion of IL-6 by liver DC with reduced DAP12 expression increased significantly by 63 ± 14% (p < 0.01; n = 3), whereas TNF-α secretion increased 91 ± 30% (p < 0.01; n = 3) and IL-12p70 increased 100 ± 48% (n = 2) compared with control siRNA-transfected liver DC. By contrast, IL-10 secretion by liver DC transfected with DAP12 siRNA decreased 40 ± 13% (p < 0.05; n = 3). However, no significant change in IL-10 secretion by DAP12-silenced splenic DC was found. Reduction of DAP12 in liver but not splenic DC reduces IL-10 production in MLR

Absence of functional DAP12 has been associated with accelerated development of autoimmune disease in NOD mice and decreased regulatory T cell (Treg) function (41). When we examined the incidence of Foxp3+CD4+ T cells in MLR cultures in which DAP12-silenced liver or spleen DC were used as stimulators, no significant effect on the incidence of Treg was observed (Fig. 4B).

**FIGURE 2.** Reduction of DAP12 enhances the expression of cell surface costimulatory and coregulatory molecules by liver DC. A, Liver or spleen DC (1 × 10^6) were transfected overnight (18 h) with either negative control (ctl) siRNA or DAP12 siRNA (400 nM). DAP12 silencing was confirmed by semiquantitative RT-PCR. *p < 0.05, **p < 0.01. B, Expression of MHC class II (I-A^b), B7 family molecules, and CCR7 was evaluated by flow cytometry gating on CD11c+ cells. Shaded histograms indicate isotype controls, gray lines indicate expression of the given marker for DC transfected with ctl siRNA, and black lines indicate expression of the given marker for DC transfected with DAP12 siRNA. C, Bar graphs show the MFI for the indicated molecule expressed on DC transfected with DAP12 siRNA normalized to the MFI for the indicated marker expressed on DC transfected with ctl siRNA + 1 SD for the markers indicated. D shows the percentage of liver DC positive for I-A^b, CD86, and CD80. Data were obtained from a minimum of three separate experiments. **p < 0.05 comparing fold change between liver and spleen DC. †indicates significant increases (*p < 0.05) in MFI for the indicated surface molecule in DAP12 siRNA-transfected DC compared with ctl siRNA-transfected DC (C). *Indicates significance of p < 0.05 comparing percentage of positive cells for the indicated surface molecule in DAP12 siRNA-transfected DC with ctl siRNA-transfected DC (D).
In contrast, significant downregulation of IL-10 secretion was observed in MLR supernatants when liver but not spleen DC with reduced expression of DAP12 were used to stimulate allogeneic T cells (Fig. 4C). These data suggest that DAP12 may regulate the ability of liver DC to induce or sustain IL-10–secreting Treg type 1 cells (60, 61), potentially mediated through DC-secreted IL-10.

**DAP12 deficiency enhances liver DC responses to LPS and their consequent T cell stimulatory capacity**

Previously, we demonstrated the inferior responses of freshly isolated liver DC to TLR ligation compared with DC from secondary lymphoid tissue (30, 32). It has also been found that DAP12 acting in concert with another ITAM-expressing adaptor, FcRγ, controls the magnitude of responses to TLR ligation in BM-DC (45). We hypothesized that silencing DAP12 would enhance liver DC maturation in response to the TLR4 ligand LPS. Following transfection with DAP12 siRNA, liver DC significantly upregulated CD80 and CD86 expression in response to LPS when compared with liver DC transfected with ctl siRNA (Fig. 5A). A similar trend was seen with splenic DC. Compared to CD4+ and CD8+ T cells cultured with LPS-stimulated ctl siRNA-transfected liver DC, those stimulated with LPS-activated, DAP12-silenced liver DC did not display enhanced proliferative capacity (Fig. 5B) but did have a more activated phenotype (CD44 hiCD62Llo) (50.4 ± 7.3% for CD4+ and 56.4 ± 19% for CD8+ T cells stimulated with DAP12-silenced liver DC compared with 37.2 ± 5.9% and 45.6 ± 21%, respectively, for ctl siRNA-transfected cells; n = 2; p < 0.05 and p < 0.01, respectively; data not shown). No difference in T cell proliferation was seen in MLR cultures in which DAP12 siRNA-treated splenic DC stimulated with LPS were used as stimulators (Fig. 5B). There was no difference in the incidence of CD4+Foxp3+ T cells in MLR when DAP12 siRNA
treated liver or spleen mDC stimulated with LPS were used as stimulators (data not shown). LPS stimulation of DAP12-silenced liver or spleen DC resulted in increased IFN-\(\gamma\) but decreased IL-4 and IL-10 production in MLR in which they were used as stimulators compared with control siRNA-treated DC (Fig. 5C). Collectively, these data suggest that DAP12 may hamper LPS-induced maturation in liver DC, thereby suppressing their allostimulatory capacity.

**IL-10 but not IL-6 blocks maturation of DAP12-silenced liver DC**

Our data show that liver DC lacking DAP12 upregulate CD80 and CD86 expression and secrete increased amounts of IL-6, TNF-\(\alpha\), and IL-12p70. These DC also produce reduced levels of the anti-inflammatory cytokine IL-10 that is known to inhibit DC maturation and function (62–64). We hypothesized that the addition of exogenous IL-10 might restore the expression of costimulatory molecules on liver DC with reduced DAP12 expression to the levels observed on ctl liver DC. When we added exogenous IL-10 or DAP12 siRNA-transfected liver but not splenic DC, the expression of CD80 and CD86 was reduced significantly (Fig. 6A). The response of liver DC with reduced DAP12 expression to exogenous IL-10 was similar to that of liver DC transfected with ctl siRNA, suggesting that DAP12 expression plays little role in the regulation of DC responses to IL-10. Like IL-10, IL-6 also blocks DC maturation (65), particularly of liver DC (34). Although exogenous IL-6 reduced the expression of CD80 and CD86 expression on liver DC transfected with ctl siRNA (Fig. 6B), neither liver nor spleen DC with reduced DAP12 downregulated CD80 or CD86 expression in response to exogenous IL-6.

**Reduction of DAP12 decreases STAT3 phosphorylation in mature liver DC**

IL-6 and IL-10 initiate transcription events through the activation of STAT3. As liver DC with reduced DAP12 expression had altered responses to IL-6 but not IL-10 compared with ctl siRNA-transfected DC, we hypothesized that IL-6–driven STAT3 activation might be altered in these cells. Liver DC that had been transfected overnight withctl or DAP12 siRNA were stimulated with either IL-6 or IL-10, then probed for p-Y705, p-S727, and total STAT3 to evaluate the activation status of STAT3. Liver DC had higher levels of p-STAT3 (both Y705 and S727) compared with splenic DC in the absence of exogenous stimulation (Fig. 6C). Both IL-6 and IL-10 increased the level of p-Y705 and p-S727 STAT3 in liver DC transfected with control siRNA. Reduction of DAP12 enhanced the basal level of STAT3 p-Y705 and p-S727 phosphorylation in liver DC but not spleen DC. Furthermore, liver DC transfected with DAP12 siRNA exhibited robust STAT3 phosphorylation in response to IL-10, but not IL-6, reflecting the changes in CD80 and CD86 expression shown in Fig. 6A and 6B.

The upregulation of STAT3 phosphorylation seen in DAP12-silenced liver DC seemed contradictory, as these cells upregulated expression of activation markers (Fig. 2B, 2C), and STAT3 phosphorylation correlates with DC inactivation (66, 67). To address this apparent paradox, liver DC transfected with ctl or DAP12 siRNA overnight were stained for p-Y705–STAT3. When total CD11c\(^+\) cells were examined, liver DC with reduced DAP12 expression again exhibited higher levels of p-Y705–STAT3 (Fig. 6D). However, more
focused analysis showed that in ctl siRNA-transfected DC, the CD11c+CD80+ or CD11c+CD86+ population expressed the highest level of p-Y705–STAT3 (Fig. 6E, 6F). Likewise, in DC transfected with DAP12 siRNA for 2 h were also stimulated with IL-6 (1 ng/ml). *Denotes a trend toward biological significance (p = 0.056). Cells were harvested as in A. Bars show means ± 1 SD for the MFI of IL-6–treated DC for the indicated marker, relative to the MFI of nontreated DC (n = 3 experiments). C, DC were transfected overnight with ctl siRNA or DAP12 siRNA, then stimulated for 20 min with either IL-6 or IL-10 (1.0 ng/ml). The cells were washed and total lysate probe for p-S727–STAT3, p-Y705–STAT3, or total STAT3. Data are representative of three independent experiments. D and E, Liver DC were transfected overnight with either ctl siRNA or DAP12 siRNA, washed, then stained for CD11c, B220, CD80, CD86, and p-Y705–STAT3. D, CD11c+B220+ DC were gated then examined for expression of Y705–STAT3. Shaded histogram represents isotype control, gray histogram represents ctl siRNA-transfected cells, and black histogram represents DAP12 siRNA-transfected cells. E, Expression of p-Y705–STAT3 is shown for either CD11c+CD80+ DC (left panels) or CD11c+CD86+ DC (right panels) (black histogram) or CD11c+CD80 (left panels) or CD11c+CD86 (right panels) (gray histogram). Isotype control is shown as shaded histogram. Percentages denote percent CD11c+ cells expressing CD80 or CD86. Data are representative of three independent experiments. F, Bars represent the mean ± 1 SD from three experiments evaluating p-Y705–STAT3 expression (MFI) in mature cells (either CD80hi or CD86hi) relative to the expression of p-Y705–STAT3 in immature (CD80lo or CD86lo) cells. *p < 0.05 comparing p-Y705–STAT3 from DC transfected with negative control or DAP12 siRNA.

FIGURE 6. Reduction of DAP12 alters STAT3-mediated responses. A, Liver or spleen DC were transfected with siRNA for 2 h prior to the addition of IL-10 (1.0 ng/ml). After overnight culture, the DC were harvested and analyzed by flow cytometry for the expression of CD86 or CD80. Vertical bars show means ± 1 SD for the MFI of IL-10–treated DC for the indicated marker relative to the MFI of nontreated DC (n = 3 separate experiments). *p < 0.05 comparing the MFI of nontreated cells to IL-10–treated cells. B, DC transfected with ctl or DAP12 siRNA for 2 h were also stimulated with IL-6 (1 ng/ml). Denotes a trend toward biological significance (p = 0.056). Cells were harvested as in A. Bars show means ± 1 SD for the MFI of IL-6–treated DC for the indicated marker, relative to the MFI of nontreated DC (n = 3 experiments). C, DC were transfected overnight with ctl siRNA or DAP12 siRNA, then stimulated for 20 min with either IL-6 or IL-10 (1.0 ng/ml). The cells were washed and total lysate probe for p-S727–STAT3, p-Y705–STAT3, or total STAT3. Data are representative of three independent experiments. D and E, Liver DC were transfected overnight with either ctl siRNA or DAP12 siRNA, washed, then stained for CD11c, B220, CD80, CD86, and p-Y705–STAT3. D, CD11c+B220+ DC were gated then examined for expression of Y705–STAT3. Shaded histogram represents isotype control, gray histogram represents ctl siRNA-transfected cells, and black histogram represents DAP12 siRNA-transfected cells. E, Expression of p-Y705–STAT3 is shown for either CD11c+CD80+ DC (left panels) or CD11c+CD86+ DC (right panels) (black histogram) or CD11c+CD80 (left panels) or CD11c+CD86 (right panels) (gray histogram). Isotype control is shown as shaded histogram. Percentages denote percent CD11c+ cells expressing CD80 or CD86. Data are representative of three independent experiments. F, Bars represent the mean ± 1 SD from three experiments evaluating p-Y705–STAT3 expression (MFI) in mature cells (either CD80hi or CD86hi) relative to the expression of p-Y705–STAT3 in immature (CD80lo or CD86lo) cells. *p < 0.05 comparing p-Y705–STAT3 from DC transfected with negative control or DAP12 siRNA.

focused analysis showed that in ctl siRNA-transfected DC, the CD11c+CD80+ or the CD11c+CD86+ population expressed the highest level of p-Y705–STAT3 (Fig. 6E, 6F). Likewise, in DC transfected with DAP12 siRNA, the CD11c+CD80+ or CD11c+CD86+ cells expressed higher levels of p-Y705–STAT3 compared with CD11c+CD80+ or CD11c+CD86+ cells (Fig. 6E, 6F). However, a greater proportion of CD11c+CD80+ DC transfected with ctl siRNA expressed p-Y705–STAT3 compared with CD11c+CD80+ DC transfected with DAP12 siRNA. The enhanced expression of p-Y705–STAT3 seen by Western blot in Fig. 6C may thus reflect an increase in the incidence of mature DC, but not an increase in STAT3 activation on a per-cell basis. Collectively, these data suggest that DAP12 may positively regulate STAT3 phosphorylation and subsequent activation of liver DC.

Loss of DAP12 reduces IRAK-M expression in liver but not splenic DC

It has been reported (34) that IL-6–driven STAT3 signaling enhances IRAK-M expression in bulk CD11c+ liver DC. We observed that IRAK-M expression was diminished markedly in DAP12 siRNA-transfected liver but not spleen DC (Fig. 7A). Addition of exogenous IL-6 further reduced IRAK-M expression in DAP12-silenced liver DC (Fig. 7B). Taken together, these data suggest that DAP12 may be essential for IL-6–driven STAT3 activation and IRAK-M expression in liver DC.

TREM-1 is expressed at higher levels by liver compared with splenic DC

The foregoing data suggest that DAP12 regulates the maturation of liver DC while exerting a lesser influence on splenic DC maturation. We hypothesized that this might reflect differences in the pairing of immune regulatory receptor(s) with DAP12 on these two DC populations. DAP12 initiates signaling from a number of receptors in myeloid cells (38), including TREM-1, TREM-2, and CD200R, that have been reported to modulate cell activation. Generally, TREM-1 triggers secretion of proinflammatory cytokines, whereas TREM-2 attenuates inflammatory responses of myeloid cells (68). CD200R expression on DC is associated with a
tolerogenic phenotype (69). A recent study reported CD200R4, but not TREM-1 or TREM-2, mRNA expression by conventional splenic DC (46). Notably, both TREM-1 and TREM-2 are expressed by hepatic macrophages (70), suggesting that the liver microenvironment may influence their expression. TREM-1 or TREM-2 expression has not been examined previously on hepatic DC. We hypothesized that the differences in maturation and function that we identified between liver and spleen DC with reduced DAP12 expression might reflect differential expression of CD200R, TREM-1, or TREM-2 by the two populations. CD200R could not be detected by flow cytometry on either freshly isolated liver or spleen DC. As shown in Fig. 8A, flow cytometric analysis revealed that TREM-1 was expressed on a much greater proportion of freshly isolated liver DC and at a 2-fold higher level (mean fluorescence intensity [MFI]) on these cells than on spleen DC. The extent of TREM-1 expression was lower than that on positive control CD11b+ peritoneal cells. TREM-2 was expressed only at low levels by both liver and spleen DC. TREM-1 ligation is activating (68), though TREM-1 ligation during the course of bacterial infection induces expression of IRAK-M and can be inhibitory (71).

**Loss of DAP12 by liver DC alters their T cell priming ability in vivo**

The allostimulatory capacity of DC with reduced DAP12 expression was enhanced significantly in vitro (Fig. 4A). To characterize the role of DAP12 in hepatic DC relative to the priming of T cell responses in vivo, we performed adoptive transfer of these cells to fully allogeneic recipients. In these experiments, liver DC (C57BL/6) were transfected in vitro with either ctl siRNA or DAP12 siRNA. Expression of IRAK-M mRNA was then evaluated by semiquantitative RT-PCR. Data are representative of three separate experiments. *p < 0.05, **p < 0.01, ***p < 0.005. AU, arbitrary units.

**Figure 7.** DAP12 silencing diminishes IRAK-M expression in liver but not splenic DC. A, Liver or spleen DC were transfected overnight with ctl siRNA or DAP12 siRNA. Expression of IRAK-M mRNA was then evaluated by semiquantitative RT-PCR. B, Liver DC were transfected for 2 h prior to the addition of IL-6 (1.0 ng/ml). Cells were collected 18 h later, and the expression of IRAK-M mRNA was evaluated by semiquantitative RT-PCR. Data are representative of three separate experiments. *p < 0.05, **p < 0.01, ***p < 0.005. AU, arbitrary units.

**Figure 8.** Liver DC express TREM-1 at higher levels than splenic DC. A, TREM-1 and TREM-2 expression was evaluated by flow analysis of freshly isolated liver or spleen DC or positive control mouse peritoneal cells (PC). Gating was on CD11c+ (for DC) or on CD11b+ (for peritoneal macrophages). Data represent the mean percent positive or MFI + 1 SD for three independent experiments. B, Data are representative of three experiments. Bars indicate means + 1 SD of triplicate values. *p < 0.05.

**Figure 9.** Liver DC with reduced DAP12 expression exhibit enhanced T cell stimulatory ability in vivo. Liver DC (2.5 × 10^6) were transfected overnight with either (−) ctl siRNA or DAP12 siRNA then adoptively transferred into normal BALB/c recipients. As shown in Supplemental Fig. 1A, T cells isolated from recipients of DAP12−/− liver DC displayed significantly (2-fold) enhanced T cell proliferative responses to C57BL/6 APC when compared with T cells isolated from recipients of wild-type liver DC. Adoptive transfer of DAP12−/− liver DC also significantly enhanced IL-4 and IFN-γ production by CD4+ and CD8+ T cells, respectively (Supplemental Fig. 1B, 1C), whereas IL-10 and IL-17 production by host T cells was not affected (data not shown). Thus, the in vivo T cell stimulatory function of liver DC is affected by DAP12 expression; DAP12 expression differentially downmodulates proinflammatory cytokine production by host T cells while also limiting the ability of liver DC to induce T cell proliferation.

**Discussion**

It is well recognized that, compared with blood-borne or lymphoid tissue DC, liver interstitial DC are comparatively immature, refractory to LPS stimulation and able to induce T cell hyporesponsiveness and promote T cell tolerance either in vitro or in vivo...
DAP12 REGULATES LIVER DENDRITIC CELL FUNCTION

Our data highlight a role for DAP12 in the regulation of IL-6–mediated STAT3 activation. DC with reduced DAP12 expression retained STAT3-mediated responsiveness to IL-10. IL-10–driven STAT3 activation is also retained in macrophages in which calmodulin-dependent protein kinase, a signaling molecule downstream of DAP12, is inhibited (76). Surprisingly, we found that DC with reduced DAP12 expression lost STAT3-mediated responses to IL-6. This could be accounted for simply as a function of IL-6R expression. Alternatively, the anti-inflammatory role of IL-6 is associated with low expression of the suppressor of cytokine signaling-3 and with a shift from IL-6–driven STAT3 to STAT1 activation (77). Such a relationship among DAP12, STAT1, and suppressor of cytokine signaling-3 expression is yet to be evaluated.

DAP12 negatively regulates activity of the tryptophan-catabolizing enzyme IDO in murine splenic DC (78). Furthermore, induction of IDO in DC requires STAT3 (79). The current findings may link these observations, delineating a potential pathway by which DAP12 inhibits the induction of IDO by altering STAT3 activity. In experiments using IDO-deficient liver DC, we have found that induction of IDO does play a role, in part, in the impaired T cell allostimulatory capacity of liver DC (T.L. Sumpter and A.W. Thomson, unpublished observations). Diminishing DAP12 expression in splenic DC from IDO-deficient mice modestly enhanced T cell proliferation. Surprisingly, diminishing DAP12 expression in liver DC from IDO-deficient mice did not further enhance their T cell allostimulatory capacity, suggesting that IDO inhibition is not a dominant function of DAP12 in liver DC (T.L. Sumpter and A.W. Thomson, unpublished observations).

The data presented in this study suggest that DAP12 is constitutively active in liver DC, as a reduction in DAP12 levels promotes their maturation. DAP12 lacks an extracellular domain and requires a receptor for activation. Our findings reveal higher expression of the DAP12-associated receptor TREM-1 on liver but not splenic DC. At present, the natural ligand for TREM-1 is unknown, although interactions between TREM-1 and LPS, which is found constitutively in the liver, have been reported (80). Furthermore, whereas TREM-1 ligation is associated with enhanced inflammatory responses, recent studies have also implicated TREM-1 in the downregulation of inflammation (81). TREM-1 ligation during the course of bacterial infection induces the expression of IRAK-M in vivo and is inhibitory (71), further suggesting the possibility that the inhibitory effects of DAP12 in liver DC may be mediated by IRAK-M. Finally, a role for TREM-1 in regulation of STAT3 signaling has been described in neutrophils (82). These reports and our finding that TREM-1 is expressed on liver DC make this triggering receptor a potential mediator of the effects ascribed to DAP12 in liver DC. Investigations into the role of TREM-1 in modulating liver DC function are underway in our laboratory.

In summary, our studies demonstrate both qualitative and quantitative differences in the expression and function of DAP12 between murine liver and splenic DC. DAP12 appears to play an important role in positive regulation of IRAK-M expression and IL-10 production by liver conventional mDC that is not evident in splenic DC. Its expression may also account, at least in part, for the inferior ability of hepatic DC to induce allogensic T cell proliferation compared with lymphoid tissue DC.

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Disclosures
The authors have no financial conflicts of interest.
References


