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The Src-Like Adaptor Protein Regulates GM-CSFR Signaling and Monocytic Dendritic Cell Maturation


GM-CSF is an important cytokine involved in myeloid differentiation and inflammatory processes. Signaling through the GM-CSFR also plays a critical role in the generation of monocyte-derived dendritic cells (DC). In this article, we report that the Src-like adaptor protein (SLAP) functions as a negative regulator of the GM-CSFR. In bone marrow-derived DC (BM-DC) lacking SLAP and the closely related SLAP2, downregulation of GM-CSFRβ was impaired, leading to enhanced phosphorylation of Jak2 and prolonged activation of Akt and Erk1/2 in response to GM-CSF stimulation. Compared with wild-type bone marrow, SLAP/SLAP2−/− bone marrow gave rise to similar numbers of CD11c+ and CD11b+ DC, but SLAP/SLAP2−/− BM-DC failed to acquire high levels of MHC class II, CD80, and CD86, indicating an impairment in maturation. Furthermore, MHC class II expression in SLAP/SLAP2−/− BM-DC was rescued by decreasing GM-CSF concentration, suggesting that enhanced GM-CSF signaling mediates the block in maturation. In addition, SLAP/SLAP2−/− BM-DC produced less IL-12 and TNF-α in response to LPS compared with controls and failed to stimulate T cells in an MLR. Ag-specific T cell activation assays showed that SLAP/SLAP2−/− BM-DC were less robust at inducing IFN-γ secretion by DO11.10 T cells. These results indicated that SLAP-mediated GM-CSFR regulation is important for the generation of functionally mature monocyte DC.

Dendritic cells (DC), professional APCs of the immune system, detect and endocytose Ag to direct T cell responses. DC are a heterogeneous group of immune cells, and each subtype is thought to have distinct functions in coordinating the immune response (1). Mature monocyte-derived DC, which develop from peripheral blood monocytes or bone marrow (BM) monocyte progenitor cells under the influence of GM-CSF, are a subset of DC that are involved in inflammatory processes and infection clearance (1–3). Monocyte-derived DC have also been used to demonstrate the ability of DC to initiate autotumor responses in vivo (4). Monocyte-derived DC are distinct from Flt3L-derived DC, which exist as steady-state DC in the spleen and in peripheral lymphoid organs (5).

Abbreviations used in this article: BM, bone marrow; BM-DC, bone marrow-derived dendritic cell; OEBP, Ccaat-enhancer binding protein; DC, dendritic cell; DKO, double knockout; MHCII, MHC class II; NP-40, Nonidet P-40; SLAP, Src-like adaptor protein; WT, wild-type.

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that SLAP2 binds to and regulates M-CSFR and CSF-1R; expression of dominant negative SLAP2 results in a potentiation of CSF-1-mediated differentiation (14, 16). Similar to the situation in T cells, these effects also seem to be mediated through c-Cbl (14). In this study, we examined the role of SLAP and SLAP2 in cytokine receptor-signaling pathways by studying GM-CSF responses in primary BM-DC. In this article, we show that SLAP functions as a negative regulator of the GM-CSFR and demonstrate that downregulation of GM-CSF signaling is important for monocytic DC maturation.

Materials and Methods

Mice

SLAP−/− BALB/c and C57BL/6 mice were generated at the Howard Hughes Institute at University of California, San Francisco, as previously described (9). SLAP−/− mice were generated by methods shown in Supplemental Fig. 1. Mice were bred and maintained under institute guidelines at the Toronto Centre for Phenogenomics, and experiments were performed on mice 6–10 wk of age. DO11.10 BALB/c mice were purchased from Jackson Laboratory (#003303). All studies and procedures were approved by the Hospital for Sick Children Animal Care and Use Committee.

Reagents and Abs

Anti-SLAP Abs were used as previously described (10). Anti-SLAP Abs were produced by immunizing rabbits with a GST-fusion protein directed against the N terminus of murine SLAP. Goat anti-SLAP (C-19), GM-CSFRα (M-20), and GM-CSFRβ (K-17) Abs were purchased from Santa Cruz Biotechnology. Allophycocyanin-conjugated CD11c (clone HL3) Ab was purchased from BD Pharmingen. FITC-conjugated Abs against CD11b (M1/70), CD86 (GL1), CD80, and MHCII (M5/114,15.2) were purchased from eBioscience. Recombinant murine GM-CSF and murine IL-4 were purchased from R&D Systems. LPS (O55:B5) was purchased by Sigma. Abs against phospho-Akt (S473), Akt, phospho-Erk1/2 (T202/Y204), Erk1/2, phospho-Jak2 (Y1007/1008), Jak2, phospho-STAT3 (Y705), Stat3, phospho-STAT5 (Y694), Stat5, Cbl, and SOCS3 were purchased from Cell Signaling Technology. Phosphorytrosine Ab (4G10) and anti-mouse β-actin (C4) were purchased from Millipore.

Generation of BM-DC

BM-DC were generated as described (3). Briefly, BM was harvested from the femurs and tibias of wild-type (WT) and SLAP/SLAP2−/− mice by flushing the marrow cavity with complete RPMI 1640 (containing 10% FBS, 50 mM 2-ME, 1% penicillin [100 U/ml], and 1% streptomycin [100 U/ml]). RBCs were lysed using an ammonium chloride lysis buffer, and remaining cells were counted on a Coulter ViCell counter. Nucleated BM cells were resuspended at a density of 1 × 10^7 cells/ml in complete RPMI 1640 containing 10% FCS and 2 mM L-glutamine. BM cells were incubated in the presence of 10 ng/ml GM-CSF and 5 ng/ml IL-4 for 6 days. After 6 days, BM cells were incubated with 1 μg/ml mitomycin C (Sigma-Aldrich) to prevent cell division. For use in experiments in which GM-CSF concentration was reduced, BM was cultured in 0.1, 1.0, 5, and 150 mM NaCl, 2 mM EGTA, 1.5 mM MgCl2, 1% [v/v] NP-40, and 10% [v/v] glycerol containing complete protease inhibitors (Roche), 10 mM sodium pyrophosphate, 100 mM NaF, and 1 mM Na3VO4.

Surface biotinylation

BM-DC were left unstimulated or stimulated for 5 and 10 min with 100 ng/ml GM-CSF at 37°C and then put on ice for 5 min. The cells were washed twice with ice-cold PBS. A total of 1 × 10^6 cells was incubated with 0.5 mg/ml EZ-link-sulfo-NHS-LC-Biotin (Pierce) in biotinylation buffer (10 mM HEPES, 154 mM NaCl, 3 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 10 mM glucose) for 1 h at 4°C to label cell-surface proteins. The cells were washed twice with ice-cold PBS and blocked for 5 min in RPMI 1640 + 10% FBS with 100 mM glucose to quench biotin. The cells were resuspended with cold PBS and then lysed in NP-40 lysis buffer. One milligram of lysate was incubated with 30 μl streptavidin-agarose beads overnight at 4°C to isolate biotinylated proteins. Streptavidin pull-downs were immunoblotted for GM-CSFRβ and transferrin receptor (used as cell-surface control) and quantified by densitometry.

IL-12 assays

Day 8–10 BM-DC were treated with protein transport inhibitor (BD GolgiStop) for 4 h prior to fixation and permeabilization with 4% paraformaldehyde and 0.2% Tween in PBS for 30 min on ice. Fixed/permeabilized cells were stained with PE-conjugated anti-IL-12 p40/p70 Ab (C15.6 BD Pharmingen). ELISA kit and protocol from R&D Systems were used to assess IL-12p40, TNF-α, and IL-6 concentrations in BM-DC supernatants 12–16 h after LPS treatment.

Isolation of splenocytes

Spleens were removed from 6–10-wk-old mice and treated with collagenase D for 20 min at 37°C prior to passage through a 70-μm cell strainer. RBCs were lysed, and the single-cell suspension was counted. A total of 1 × 10^7 cells was incubated with 1 μg/ml FcBlock (anti-CD16/32) prior to staining with fluorochrome-conjugated Abs for analysis by flow cytometry.

T cell-proliferation assays

BM-DC were treated with 1 μg/ml LPS (O5:B5) for 12–16 h, counted, and treated with mitomycin C (Sigma-Aldrich) to prevent cell division. For the MLR, T cells were enriched from splenocytes isolated from C57BL/6 and BALB/c mice with the CD3+ mouse T cell enrichment kit ( Stem Cell Technologies). A total of 1 × 10^7 T cells was cultured with 100, 1000, or 1 × 10^5 cells/ml in each well of a 96-well plate. On day 4 of culture, 1 μCi [3H]thymidine was added to each well for an additional 6 h of culture, cells were transferred onto filters using an automated cell harvester, and β-counts were measured on a Coulter Scintillation counter. For in vitro immune-response assays, 1 × 10^4 LPS-treated BM-DC were pulsed with 10^-5 M OVA253–269 peptide for 4 h and then cocultured with 1 × 10^5 DO11.10 T cells, which were isolated from the spleens of DO11.10 mice using the EasySep CD4+ T cell enrichment kit (Stem Cell Technologies). WT and SLAP/SLAP2−/− BM-DC and CD4+ DO11.10 T cells were cocultured in RPMI 1640 + 10% FBS for 3 d in a 96-well flat-bottom plate. Cell supernatants were removed, and IFN-γ and IL-4 concentrations were measured by ELISA (R&D Systems).

Statistical analysis

The two-tailed Student t test was used to determine statistical significance. A p value < 0.05 was considered statistically significant.

Results

GM-CSFRβ downregulation is impaired in SLAP/SLAP2−/− BM-DC

SLAP and SLAP2 are expressed in many hematopoietic cell types and have a negative regulatory role in the regulation of TCR, BCR,
and CSF-1R (8–16). Our previous studies showed a role for SLAP2 in CSF-1R signaling, leading us to examine whether SLAP and SLAP2 could be involved in the regulation of hematopoietic cytokine signaling and early myeloid differentiation. We chose to examine the role of SLAP and SLAP2 in GM-CSFR signaling in DC because GM-CSF is a cytokine involved early in myeloid differentiation and DC development (20, 21).

To examine SLAP and SLAP2 expression in BM-DC, we determined mRNA levels during differentiation of BM in GM-CSF and IL-4 over 9 d. SLAP and SLAP2 mRNA was detected in BM and BM-DC grown under these conditions (Fig. 1A). Protein expression was examined in lysates from WT and SLAP/SLAP2−/− BM-DC by immunoblotting with Abs against SLAP and SLAP2. Thymus lysates from WT and SLAP/SLAP2−/− mice were used as a positive control. SLAP protein was detected in WT thymus and BM and BM-DC lysates; however, SLAP2 protein levels were not detectable by Western blot (Fig. 1B, data not shown). Although SLAP2 protein was not detectable in mature DC, we chose to perform experiments using BM from mice deficient in SLAP and SLAP2 to exclude the possibility of functional redundancy in early DC progenitors.

SLAP-deficient mice have documented developmental defects in the T and B cell hematopoietic lineages (9, 13). SLAP2 knockout mice were generated but do not display the same B or T cell abnormalities as SLAP−/− mice (L. Dragone, L. Shaw, C. White, and A. Weiss, unpublished observations). We crossed SLAP−/− and SLAP2−/− mice to generate mice null for both proteins. SLAP/SLAP2 double-knockout (DKO) mice do not display any gross abnormalities and have normal hematological parameters (Supplemental Table I). BM from WT and SLAP/SLAP2−/− mice was cultured in GM-CSF and IL-4 to derive DC. We investigated whether SLAP and SLAP2 deficiency had any effect on the GM-CSF–signaling pathway by stimulating day 9 BM-DC for 5, 10, and 20 min with GM-CSF. In response to ligand, we observed an increase in tyrosine phosphorylated proteins in SLAP/SLAP2−/− BM-DC compared with WT cells (Fig. 1C). In WT BM-DC, GM-CSFR levels were reduced 2–3-fold after 20 min of stimulation, indicating that the receptor undergoes downregulation upon activation with ligand (Fig. 1D). In contrast, GM-CSFR levels in SLAP/SLAP2−/− BM-DC were 3–4-fold greater at steady-state (0 time point); although they decreased slightly following stimulation, they remained 4-fold higher than WT receptor levels at 20 min following ligand stimulation (Fig. 1D). GM-CSFR levels were similar in WT and SLAP/SLAP2−/− BM-DC and did not decrease in response to GM-CSF (Fig. 1D).

Because total GM-CSFR levels in SLAP/SLAP2−/− BM-DC lysates did not decline following stimulation with GM-CSF compared with WT, cell surface receptor levels were investigated by labeling with biotin. BM-DC were stimulated with GM-CSF for 5 and 10 min or were left unstimulated, followed by incubation on ice to halt endocytosis and labeling with biotin with at 4°C. Streptavidin–agarose beads were used to pull down tyrosinated surface proteins by centrifugation. The amount of GM-CSFR on the cell surface prior to GM-CSF stimulation was comparable in SLAP/SLAP2−/− and WT BM-DC (Fig. 2A, left panels). After 5 and 10 min of stimulation with GM-CSF, an average of 32 ± 15% and 22 ± 14% of initial GM-CSFR remained on the cell surface of WT BM-DC, respectively, whereas 78 ± 8% of initial GM-CSFR remained on the cell surface of SLAP/SLAP2−/− BM-DC after 10 min of stimulation (Fig. 2A, left panels, Fig. 2B). The broadening of the GM-CSFR protein band, seen after 5 and 10 min of stimulation in the SLAP/SLAP2−/− BM-DC lysates, was most likely due to accumulation of tyrosine phosphorylated GM-CSFR. These results indicated that ligand-induced downregulation of cell-surface GM-CSFR is impaired in SLAP/SLAP2−/− BM-DC. Previous studies from our group and other investigators showed that SLAP and SLAP2 mediate downregulation of AgRs through interaction with the E3 ubiquitin-ligase c-Cbl (10, 12). Attempts to coimmunoprecipitate Cbl and GM-CSFR in BM-DC were unsuccessful; however, Cbl protein levels in SLAP/SLAP2−/− BM-DC lysates were elevated compared with WT BM-DC (Fig. 2C), suggesting that a change in Cbl function may underlie the failure to downregulate GM-CSFR in these cells.

GM-CSFR signaling was shown to activate STAT3 and STAT5 downstream of Jak2 phosphorylation, as well as members of the PI3K and MAPK pathways (22). To determine whether impaired downregulation of GM-CSFR in SLAP/SLAP2−/− BM-DC led to alterations in the activation of downstream signaling pathways, the phosphorylation status of Jak2, STAT3, STAT5, Akt, and Erk1/2 was examined. Comparison of total cell lysates from WT and SLAP/SLAP2−/− BM-DC revealed that at 5 min following GM-CSF stimulation, Jak2 phosphorylation was 2–6-fold higher in SLAP/SLAP2−/− BM-DC compared with WT controls, and it remained higher at 10 and 20 min (Fig. 3A). In addition, total Jak2 protein levels in SLAP/SLAP2−/− DC were half of WT levels (densitometry quantification, WT: 1.1 ± 0.13, DKO: 0.51 ± 0.21; n = 8) (Fig. 3A). Phosphorylation of Akt and Erk1/2 in response to
SLAP REGULATION OF GM-CSFR AND BM-DC MATURATION

GM-CSF was prolonged in SLAP/SLAP2−/− BM-DC in comparison with WT cells (Fig. 3B). Akt phosphorylation was 2-fold higher at 10 and 20 min following stimulation, whereas Erk phosphorylation was 2-fold higher at 10 min (Fig. 3B). These results showed that GM-CSFR signaling is deregulated in the absence of SLAP and SLAP2, leading to enhanced and sustained activation of a subset of downstream-signaling molecules in response to GM-CSF. Despite the apparent hyperphosphorylation of Jak2, STAT3 and STAT5 phosphorylation in SLAP/SLAP2−/− BM-DC was similar to that in WT DC and, thus, may be regulated by other mechanisms in the presence of abnormally activated GM-CSFR or maximally activated in WT cells.

SLAP interacts with the α subunit of the GM-CSFR
SLAP and SLAP2 contain an SH2 domain that mediates interactions with several tyrosine-phosphorylated proteins involved in the TCR-signaling pathway. To determine whether SLAP and SLAP2 interact with tyrosine-phosphorylated proteins in BM-DC, day 9 in vitro-differentiated cells were depleted of cytokines and then stimulated with GM-CSF for 5 min. Stimulated and unstimulated protein cell lysates were incubated with GST-fusion proteins of full-length SLAP and full-length SLAP2 that were purified from bacterial lysates. In addition, an SH2 and SH3 point mutant of SLAP, R111K, and P73L were also produced to determine whether binding is dependent on these protein-interaction motifs (Fig. 4A). Several tyrosine-phosphorylated proteins were detected binding to GST-SLAP, but not GST-SLAP2, after stimulation of BM-DC with GM-CSF (Fig. 4B). To verify whether GM-CSFRβ and/or GM-CSFRα were associated with SLAP, the GST pull-downs were immunoblotted with Abs against both proteins. We were unable to detect a strong interaction of SLAP with GM-CSFRβ; however, we consistently observed binding of SLAP to GM-CSFRα (Fig. 4B, data not shown). The interaction seemed to be dependent on an intact SH3 and SH2 domain, because the P73L and R111K mutant forms of SLAP failed to bind to GM-CSFRα.

SLAP and SLAP2 deficiency results in BM-DC with decreased MHCII expression
To examine the consequence of deregulated GM-CSF signaling, we analyzed BM-DC from SLAP/SLAP2−/− mice by flow cytometry 8–10 d after plating. WT and SLAP/SLAP2−/− BM produced similar numbers of DC that were 70–90% positive for the myeloid DC markers CD11c and CD11b (WT: 85.1 ± 5.9%, DKO: 83.9 ± 6.46%) (Fig. 5A). In contrast, the percentage of MHCII+CD11c+ BM-DC from SLAP/SLAP2−/− mice was less than half that of WT (WT: 55.97 ± 7.1%, DKO: 17.33 ± 9.35%; p = 0.0006) (Fig. 5A). Analysis of splenic DC subsets from SLAP/SLAP2−/− mice revealed a slightly lower percentage of CD11c+ cells, despite increased spleen cellularity; however, the percentage of MHCII+ cells relative to CD11c+ cells was similar between WT and SLAP/SLAP2−/− mice (Supplemental Fig. 2). These results indicated that MHCII expression is unaffected in the steady-state resident DC of the spleen of SLAP/SLAP2−/− mice, whereas MHCII expression in the monocytic subset of DC, arising from BM progenitors in response to GM-CSF and IL-4, is impaired.

Immature DC express MHCII within an intracellular endosomal compartment that traffics to the plasma membrane upon a maturation signal (23). To determine whether the reduced surface expression of MHCII observed in SLAP/SLAP2−/− BM-DC was due to a failure in MHCII trafficking to the plasma membrane, we stained BM-DC for MHCII by immunofluorescence. SLAP/SLAP2−/− BM-DC had an overall decrease in the number of MHCII+ cells, with and without LPS, compared with WT BM-DC, indicating that MHCII expression and not localization is impaired in these cells (Supplemental Fig. 3).

Reducing GM-CSF concentration rescues MHCII expression in SLAP/SLAP2−/− BM-DC
Because GM-CSFR signaling normally promotes maturation, yet we observed impaired MHCII expression, we postulated that hyperactive GM-CSF signaling might have a negative effect on BM-DC maturation. To examine whether reducing GM-CSF signaling could alter MHCII expression in these cells, BM was cultured with a fixed concentration of IL-4 and decreasing concentrations of GM-CSF (10, 5.0, 1.0, and 0.1 ng/ml). As expected, low concentrations (0.1 and 1.0 ng/ml) of GM-CSF resulted in lower yields of CD11b+CD11c+ BM-DC compared with the use of high concentrations (Fig. 5B, 5C). However, the percentage of MHCII+expressing cells in SLAP/SLAP2−/− BM-DC was similar to WT BM-DC at
the lowest concentration (0.1 ng/ml) of GM-CSF (WT: 89 ± 7.3%, DKO: 83.2 ± 6.6%). At increasing GM-CSF concentrations, SLAP/SLAP2−/− BM-DC exhibited a loss of MHCII expression, whereas MHCII expression in WT BM-DC decreased slightly or remained the same (Fig. 5B, 5C). These results showed that hyperactivated GM-CSF signaling, as a result of SLAP and SLAP2 loss, can negatively regulate MHCII expression in BM-DC.

Because MHCII expression was restored in SLAP/SLAP2−/− BM-DC cultured at low concentrations of GM-CSF, we examined whether Jak2 phosphorylation was also normalized under these conditions. BM-DC were cultured for 9 d in 1 or 10 ng/ml of GM-CSF and IL-4 for 4 h prior to stimulation with 100 ng/ml of GM-CSF. BM-DC lysates were examined for Jak2 phosphorylation and protein expression. In the presence of 10 ng/ml of GM-CSF, SLAP/SLAP2−/− BM-DC expressed low levels of Jak2 that is enhanced in tyrosine phosphorylation status compared with WT, similar to the observations in Fig. 3. However, when SLAP/SLAP2−/− BM-DC were cultured at a lower concentration of GM-CSF (1 ng/ml), Jak2 protein levels and Jak2 phosphorylation were similar to WT BM-DC (Fig. 6). SOCS1 and SOCS3 are known regulators of JAK-STAT signaling that are induced by various cytokines, including GM-CSF and IL-4 (24). To determine whether SOCS3 might be regulating Jak2 levels in SLAP/
SLAP2−/− BM-DC, lysates from BM-DC cultured in 10 or 1 ng/ml of GM-CSF were analyzed for SOCS3 expression by Western blot. SOCS3 expression was detected in SLAP/SLAP2−/− lysates from cells cultured in 10 ng/ml of GM-CSF but not in WT BM-DC lysates or SLAP/SLAP2−/− lysates from cells cultured in 1 ng/ml of GM-CSF (Fig. 6). These data indicated that SOCS3 is upregulated in response to deregulated GM-CSF signaling in SLAP/SLAP2−/− BM-DC and may be involved in degradation of activated Jak2 under these conditions.

**BM-DC maturation is impaired in the absence of SLAP and SLAP2**

Factors that enhance maturation of BM-DC include TLR agonists, such as LPS, as well as some cytokines, particularly IL-4 (6, 25, 26). To determine how these agents promote maturation in SLAP/SLAP2−/− BM-DC, we cultured cells for 9 d in GM-CSF alone (Fig. 7A) or in GM-CSF with IL-4 (Fig. 7B) or GM-CSF with IL-4 and LPS (12 h) (Fig. 7C). BM cultured in GM-CSF with IL-4 led to a 40% increase in cell recovery of CD11c+CD11b+ BM-DC compared with cultures grown in GM-CSF alone; this was similar in SLAP/SLAP2−/− and WT cultures (data not shown). In cultures grown in GM-CSF alone, there was a slight reduction in the percentage of MHCII+ expressing SLAP/SLAP2−/− BM-DC compared with WT BM-DC (WT: 41.2 ± 8.2%, DKO: 25.7 ± 6.8%; p = 0.065) (Fig. 7A). In addition to MHCII, a proportion of mature DC expresses the costimulatory molecules CD86 and CD80. SLAP/SLAP2−/− BM-DC expressed similar levels of CD86 (WT: 8.5 ± 3.6%, DKO: 6.8 ± 4.5%) and CD80 (WT: 20.9 ± 7.0%, DKO: 14.6 ± 6.2%) as did WT BM-DC in GM-CSF alone (Fig. 7A). In the presence of GM-CSF alone, we observed that the majority of BM-DC are immature, expressing low levels of MHCII, CD80, and CD86.

In accordance with IL-4 promoting maturation of BM-DC, we observed an increase in MHCII expression (GM-CSF: 41.2 ± 8.2%, GM-CSF/IL-4: 60.9 ± 12.3%) in WT cells. In contrast, SLAP/SLAP2−/− BM-DC did not upregulate MHCII, and expression declined slightly or remained the same in response to IL-4 (GM-CSF: 25.7 ± 6.8%, GM-CSF/IL-4: 12.8 ± 6.1%) (Fig. 7B). In response to IL-4, expression of CD86 and CD80 in WT cells increased 3–5-fold (CD86: 50.3 ± 15.7%, CD80: 59.5 ± 12.5%) compared to in the presence of GM-CSF alone (Fig. 7B); however, similar to our observations with MHCII, CD80 and CD80 expression in SLAP/SLAP2−/− BM-DC did not increase in response to IL-4 (CD86: 5.7 ± 1.4%, CD80: 18.2 ± 10.6%). BM-DC were incubated overnight with LPS to determine whether maturation could be induced in SLAP/SLAP2−/− BM-DC that were differentiated in the presence of GM-CSF and IL-4. Indeed, MHCII, CD86, and CD80 expression increased in response to LPS in WT and SLAP/SLAP2−/− BM-DC; however, expression of all maturation markers in SLAP/SLAP2−/− BM-DC remained lower than that seen in WT cells (Fig. 7C). Similar increases were observed for CD80 and CD86 in SLAP/SLAP2−/− BM-DC, but, again, expression was impaired compared with WT BM-DC treated with LPS (Fig. 7C). These data suggest that, in the absence of SLAP and SLAP2, there is a block in the BM-DC maturation in response to IL-4 that can be partially, but not completely, recovered with LPS.

**SLAP loss affects IL-12 and TNF-α production by BM-DC**

Mature DC produce cytokines that are involved in the recruitment and activation of other immune cells. For example, IL-12 produced by DC is involved in the induction of Th1 differentiation of Th cells (25). In addition to acquiring MHCII and costimulatory molecules after LPS treatment, DC produced from BM in the presence of GM-CSF and IL-4 produce IL-12 (25, 26). We examined IL-12 production by WT and SLAP/SLAP2−/− BM-DC to determine whether impaired BM-DC maturation was associated with decreased IL-12 production. IL-12p40/p70 was detected using intracellular flow cytometry. Compared with WT BM-DC, IL-12 production was reduced in SLAP/SLAP2−/− BM-DC grown in GM-CSF alone or GM-CSF plus IL-4 (Fig. 8A). Similar to what was observed with MHCII expression, the difference in IL-12 production was more pronounced in the presence of IL-4. IL-12p40, TNF-α, and IL-6 secretion by WT and SLAP/SLAP2−/− LPS-treated BM-DC was measured by ELISA. IL-12p40 and TNF-α production by SLAP/SLAP2−/− BM-DC was significantly reduced compared with WT BM-DC, whereas IL-6 production was similar in WT and SLAP/SLAP2−/− cells (Fig. 8B).

DC are the primary stimulators of lymphocyte proliferation (27); therefore, we tested whether SLAP/SLAP2−/− BM-DC performed as well as WT BM-DC in stimulating T cell proliferation in an MLR. BM-DC were treated overnight with LPS prior to incubation with CD3-enriched cells from the spleens of allogeneic (C57/BL6) donors. On day 4 after T cell/BM-DC coculture, [3H]thymidine was added to the cultures, and counts were determined 6 h later. T cells incubated with SLAP/SLAP2−/− BM-DC did not proliferate to the same extent as did those cocultured with WT BM-DC (Fig. 8C). To investigate Ag-specific T cell activation by BM-DC, CD4+ T cells were isolated from the spleens of DO11.10 mice and cocultured with WT and SLAP/SLAP2−/− BM-DC that
were treated with LPS and pulsed with OVA323–339 peptide. On day 3, cell supernatants were collected, and IFN-γ and IL-4 production by DO11.10 T cells was analyzed by ELISA. Under these conditions, T cells cultured with SLAP/SLAP2−/− BM-DC produced 50–70% (60.2 ± 6.6%; n = 4) less IFN-γ than did those cultured with WT BM-DC (Fig. 8D). IL-4 production by T cells cultured with WT and SLAP/SLAP2−/− BM-DC was undetectable in these in vitro assays (data not shown). These results indicated that SLAP/SLAP2 loss impairs the ability of GM-CSF/IL-4–derived BM-DC to stimulate T cell proliferation and produce robust IFN-γ responses.

**Discussion**

GM-CSF is a hematopoietic cytokine involved in the proliferation, survival, and differentiation of myeloid cells (20, 21). GM-CSF also plays a role in pathological processes, such as inflammatory arthritis and juvenile myelomonocytic leukemia (21, 28). The processes regulating GM-CSFR downregulation are not well understood; however, modulating GM-CSF signaling through receptor downregulation may be of therapeutic benefit in pathological processes involving GM-CSF signaling. Our studies identified the Src-like adaptor proteins SLAP and SLAP-2 as negative regulators of GM-CSFR–mediated signaling.

Other studies showed that SLAP and SLAP2 act by linking receptors with c-Cbl, leading to ubiquitin-dependent downregulation (10–12). Although c-Cbl has not been identified as a negative regulator of GM-CSFR, c-Cbl is tyrosine phosphorylated after GM-CSFR engagement (29), suggesting that it plays a role downstream of receptor activation. We considered that in the absence of SLAP, an association between c-Cbl and GM-CSFRβ might be diminished; however, we were unable to detect GM-CSFR and Cbl coimmunoprecipitation. Failure to detect c-Cbl–GM-CSFR binding may be due to the transient nature of the interaction, or it could indicate the involvement of another E3 ubiquitin ligase. However, we observed increased expression of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC. Degradation of Cbl protein in SLAP/SLAP2−/− BM-DC.
following EGFR and Src activation (31–33). Therefore, the increase in c-Cbl protein levels in \( \text{SLAP}^{\text{−/−}} \) \( \text{BM-DC} \) may reflect a failure of Cbl to couple to GM-CSFR, which, in turn, would lead to accumulation of GM-CSFR\(b\) and stabilization of Cbl.

Although many SLAP- and SLAP2-binding partners have been mapped to the SH2 domain (9–14), we found that SLAP interacted with the \( \alpha \) subunit of GM-CSFR, which, in the mouse, does not contain any potential tyrosine-phosphorylation sites within the cytoplasmic tail. However, GM-CSFR\(\alpha\) contains a PXXP motif that may mediate SH3 domain interactions (34, 35). A point mutation in the SH3 domain of SLAP diminished the interaction with GM-CSFR\(\alpha\), suggesting that is a likely mode of interaction.

A recent study by Perugini et al. (36) showed that the SH3 domain of Src and Lyn bind to GM-CSFR\(\alpha\). Given that the SH3 domain of SLAP is most similar to the SH3 domain of the Src family kinases, SLAP might also compete with Src and/or Lyn for binding at this site within GM-CSFR\(\alpha\). IL-3R and IL-5R share the \( \beta \) subunit with GM-CSFR but have a unique \( \alpha \)-chain. IL-3R\(\alpha\) and IL-5R\(\alpha\) contain a PXXP motif within their \( \alpha \)-chains; therefore, it will be interesting to determine whether SLAP and/or SLAP2 has a role in the regulation of multiple cytokine receptors.

Our results revealed that in the setting of hyperactive GM-CSF signaling in BM-DC lacking SLAP and SLAP2, there is a failure of BM-DC to fully mature. In comparison with WT BM-DC, our study showed that \( \text{SLAP}^{\text{−/−}} \) \( \text{BM-DC} \) failed to produce equivalent amounts of IL-12 and TNF-\(\alpha\), are less efficient in stimulating T cell proliferation in an MLR assay, and have reduced Th1 responses in vitro. These data suggested that SLAP/SLAP\(2^{−/−}\) BM-DC are unable to progress to a more mature cell type and, thus, are unable to induce robust Th1 immune responses.

In addition, T cells cultured with WT and \( \text{SLAP}^{\text{−/−}} \) \( \text{BM-DC} \) failed to secrete IL-4 or IL-17, suggesting that, in vitro, T cell differentiation is not skewed to Th2 or Th17 by SLAP/SLAP\(2^{−/−}\).

**FIGURE 6.** Jak2 protein levels are normalized in \( \text{SLAP}^{\text{−/−}} \) \( \text{BM-DC} \) grown in low concentrations of GM-CSF. BM-DC were cultured in 10 or 1 ng/ml of GM-CSF in the presence of 5 ng/ml of IL-4. Day 9 BM-DC were depleted of GM-CSF and IL-4 for 4 h prior to stimulation with 100 ng/ml of GM-CSF for 5 min. Lysates were run out by SDS-PAGE, and Western blots were used to determine Jak2 phosphorylation status (pYJAK2), Jak2 protein level, and SOCS3 expression. Tubulin was used as a control for protein loading. Three independent experiments confirmed expression of SOCS3 in lysates from \( \text{SLAP}^{\text{−/−}} \) \( \text{BM-DC} \) grown in 10 ng/ml of GM-CSF.

**FIGURE 7.** Maturation is impaired in \( \text{SLAP}^{\text{−/−}} \) BM-DC. BM was grown in the presence of 10 ng/ml of GM-CSF alone (A), GM-CSF plus 5 ng IL-4 (B), or GM-CSF, 5 ng IL-4, plus 12 h of LPS and then stained with the following Abs: allophycocyanin-CD11c (x-axis) and unstained (I), FITC-CD11b (II), FITC-MHCII (III), FITC-CD86 (IV), FITC-CD80 (V) (y-axis) to assess maturation status. Bar graphs on the right show the average expression ± SD of each marker in three independent experiments. *\(p\) ≤ 0.1. **\(p\) ≤ 0.05.
BM-DC (data not shown). However, these data do not preclude the possibility that in vivo, SLAP/SLAP2 deficiency may affect differentiation of Th cell populations.

There are several pathways by which DC undergo maturation and become potent inducers of T cell-mediated immunity. TLR agonists, such as LPS and Cpg, are well-known stimulators of DC maturation, and cytokines, such as TNF-α and IL-4, have been used in potentiating maturation. GM-CSF was demonstrated to be a positive regulator of BM-DC maturation (37, 38); however, we showed that it can act as an inhibitor in the setting of deregulated

**FIGURE 8.** SLAP/SLAP2−/− BM-DC exhibit reduced IL-12 and TNF-α secretion and fail to stimulate T cell proliferation. A, BM-DC grown in GM-CSF or GM-CSF plus IL-4 were assessed for IL-12 production by intracellular flow cytometry 12 h after incubation with LPS. B, IL-12p40, TNF-α, and IL-6 concentrations in BM-DC cell supernatants were measured by ELISA 12–16 h after addition of LPS. Assays were performed in triplicate (mean ± SD), with at least four biological replicates. Graphs show a representative experiment. C, MLR was performed using BALB/c WT and SLAP/SLAP2−/− (DKO) BM-DC, with the number of BM-DC shown per well. The dpm were recorded, and Adpm was calculated by subtracting the dpm of BALB/c (control) T cells from the dpm of BL6 T cells. Graph shows a representative experiment (mean ± SD); three biological replicates showed similar results. D, CD4+ DO11.10 T cells were cultured with LPS-treated, OVA323–339-loaded WT and DKO BM-DC for 3 d. Cell supernatants were collected and analyzed for IFN-γ secretion by ELISA. Assay was performed in triplicate, and a representative experiment is shown. Four biological replicates yielded similar results. **p ≤ 0.05.
signaling. Increased GM-CSF signaling seemed to be the primary mediator of the block in maturation of SLAP/SLAP2−/− BM-DC, because a reduction in the concentration of GM-CSF in the media restored MHCII expression to similar levels as in WT BM-DC. Induction of negative regulators is important for temporal regulation of signaling during differentiation. SLAP expression may be temporally regulated such that it plays a critical role in regulating GM-CSF during GM-CSF– and IL-4–mediated BM-DC differentiation and maturation. Indeed, temporal regulation of SLAP is seen during T cell development, such that it regulates the TCR specifically during the double-positive (CD8+CD4+) stage of thymocyte development (9). In this way, SLAP may act in a negative feedback loop in which GM-CSF signaling induces SLAP expression, which, in turn, downregulates GM-CSF, allowing the next stage of BM-DC maturation to proceed.

In the absence of SLAP/SLAP2, BM-DC failed to acquire similar levels of CD86 and CD80 as their WT counterparts. This phenotype was more frequent in the presence of IL-4, suggesting that, in the absence of SLAP/SLAP2, IL-4 signaling does not promote upregulation of MHCII, CD80, and CD86. This might reflect a disruption in cross-talk between the GM-CSF and IL-4 pathways or a direct effect of SLAP/SLAP2 deficiency on IL-4 signaling. For example, it was shown that GM-CSF and IL-4 act synergistically in the activation of STAT1 and that this can negatively regulate CD86 at an early stage of BM-DC differentiation (24). However, in our studies, we did not detect STAT1 activation after GM-CSF stimulation (data not shown). Although we cannot exclude the possibility that SLAP regulates components of the IL-4 pathway, we were unable to detect interaction of SLAP with IL-4R (data not shown).

A similar block in GM-CSF–induced DC maturation was reported in BM-DC from Lyn−/− mice (39); however, unlike SLAP/SLAP2−/− mice, Lyn−/− mice produce increased numbers of BM-DC in response to GM-CSF. The balance between the ability of GM-CSF to drive cellular proliferation and differentiation or maturation of BM-DC likely depends on the downstream signaling molecules activated. We observed increased Akt and Erk1/2 phosphorylation but not enhanced STAT5 or STAT3 activation, suggesting that particular subsets of signaling proteins are deregulated in the absence of SLAP.

Although Jak2 activation was increased in SLAP/SLAP2−/− BM-DC, this did not correspond to an increase in STAT5 or STAT3 activation. This may be due to a compensatory response to chronic deregulated GM-CSF signaling in SLAP/SLAP2−/− BM-DC that leads to the upregulation of negative regulators of STAT signaling. Indeed, we found that SOCS3, which was shown to inhibit GM-CSF and IL-4 pathways or a direct effect of SLAP/SLAP2 deficiency on IL-4 signaling. For example, it was shown that GM-CSF and IL-4 act synergistically in the activation of STAT1 and that this can negatively regulate CD86 at an early stage of BM-DC differentiation (24). However, in our studies, we did not detect STAT1 activation after GM-CSF stimulation (data not shown). Although we cannot exclude the possibility that SLAP regulates components of the IL-4 pathway, we were unable to detect interaction of SLAP with IL-4R (data not shown).

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What is the mechanism by which deregulated GM-CSF signaling leads to impaired maturation in BM-DC lacking SLAP? One possibility is that GM-CSF signaling above a certain threshold represses transcriptional activators of MHCII and costimulatory molecules. IRF are involved in maturation and differentiation of DC. In particular, loss of IRF4 leads to impaired MHCII and CD80 expression in response to GM-CSF, whereas loss of IRF8 has similar effects on Flt3L-generated BM-DC (7). However, we did not detect a change in expression of IRF4 in SLAP/SLAP2−/− BM-DC (data not shown), indicating that this is not the mechanism by which deregulated GM-CSF leads to impaired maturation. The Ccaat-enhancer binding protein (C/EBP) family of transcriptional factors is involved in myeloid differentiation and G-CSF and GM-CSF responses (41, 42). Class II transactivator, the main regulator of MHCII gene expression, has four distinct promoters, two of which have binding sites for C/EBPβ and δ (43). In addition, C/EBPβ has been identified as a negative regulator of LPS–mediated IL-12p40 secretion in BM macrophages (44). Therefore, the C/EBP family members may play a role in suppressing BM-DC maturation in the setting of hyperactive GM-CSF signaling.

In conclusion, our results revealed a novel role for SLAP in GM-CSFR regulation. We also showed that downregulation of GM-CSFR is required for monocyte-derived DC maturation. These findings have potential implications for the role of SLAP in monocyte recruitment to the DC lineage that occurs in inflammatory processes and in response to pathogens.

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Disclosures

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References


