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Increased F4/80^{hi} macrophages is associated with suppression of serum transfer induced arthritis in mice with Flip reduced in myeloid cells

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Abstract

Objective—Macrophages are critical in the pathogenesis of rheumatoid arthritis (RA). We recently demonstrated that FLIP (FLICE-like inhibitory protein) is necessary for the differentiation and/or survival of macrophages. We also identified that FLIP is highly expressed in RA synovial macrophages. This study was performed to determine if the reduction of Flip in macrophages would reduce synovial tissue macrophages and ameliorate serum transfer induced arthritis (STIA).

Methods—Mice with *Flip* deleted in myeloid cells (*Flip^{fl/fl}LysM^{c/+}* mice) and littermate controls were employed. STIA was induced by intraperitoneal injection of K/BxN serum. Arthritis was evaluated by clinical score and change in thickness of the ankles. Joints were examined by histology and immunohistochemistry. Cells were isolated from the ankles and bone marrow and examined by flow cytometry, qRT-PCR or Western blot.

Results—In contrast to expectations, *Flip^{fl/fl}LysM^{c/+}* mice developed more severe arthritis early in the clinical course, however the peak arthritis was attenuated and the resolution phase more complete. Prior to the induction of STIA, tissue resident macrophages were reduced. Further at day 9 post arthritis induction the number of F4/80^{hi} macrophages in the joints of the *Flip^{fl/fl}LysM^{c/+}* mice was not decreased, but increased. *Flip* was reduced in the F4/80^{hi} macrophages in the ankles of the *Flip^{fl/fl}LysM^{c/+}* mice, while F4/80^{hi} population expressed an anti-inflammatory phenotype in both the *Flip^{fl/fl}LysM^{c/+}* and control mice.

Conclusions—These observations suggest that reduction of FLIP in macrophages, by increasing the number of anti-inflammatory macrophages, may be an effective therapeutic approach to suppress inflammation, depending upon the disease stage.

INTRODUCTION

Macrophages are critical to the pathogenesis of rheumatoid arthritis (RA). Macrophage numbers are increased in the synovial tissues of patients with RA (1, 2) and increased numbers of synovial tissue macrophages predict future joint damage (3). Further, the reduction of subsynovial lining macrophages observed after the initiation of therapy in patients with RA correlated with clinical benefit, while there was no correlation with other cell types such as B or T lymphocytes (4). RA synovial tissue macrophages express increased levels of pro-inflammatory chemokines and cytokines such as TNF α and IL-6, and neutralization of these cytokines is effective therapy in patients with RA (1, 2). Even though the local environment of the RA joint appears pro-apoptotic because of increased reactive oxygen and nitrogen species, RA synovial macrophages appear resistant to apoptotic cell death (5, 6). Previously we demonstrated that RA synovial macrophages express both Fas and FasL (6). They also highly express FLIP (FLICE-like inhibitory protein), which is critical for protecting RA synovial macrophages from Fas mediated apoptosis (6). Together these observations suggest that regulating the level of FLIP in macrophages might be an effective therapeutic approach to reduce the number of macrophages in patients with RA and other forms of inflammatory arthritis. Of relevance to this possibility, *Flip* (*CFLAR*) has recently been identified as an RA risk allele (7).

To examine the *in vivo* role of FLIP in macrophages, we generated genomically modified mice with the *Flip* gene floxed (8). Mice with one allele of *Flip* deleted and the other floxed (*Flip*^{f/d}) were crossed with *LysM*^{cre} (*LysM*^{c/+}) mice to ensure the optimal depletion of the floxed allele in myeloid cell lineage (8). The *Flip*^{f/d}*LysM*^{c/+} mice developed severe postnatal growth retardation and premature death, leukocytosis with multi-organ neutrophil infiltration, severe reduction of macrophages in peripheral organs together with increased extramedullary myelopoiesis (8). We were unable to detect *Flip* deficient macrophages and bone marrow progenitor cells failed to differentiate into macrophages (8) *in vitro* when *Flip* was deleted. These observations demonstrate that Flip is necessary for macrophage differentiation and survival (8).

Since the *Flip*^{f/d}*LysM*^{c/+} mice were severely ill, we generated *Flip*^{f/f}*LysM*^{c/+} mice which resulted in a less extreme phenotype. *Flip*^{f/f}*LysM*^{c/+} mice were employed to generate serum transfer induced arthritis (STIA) employing anti-glucose 6 phosphate isomerase (anti-GPI) positive serum from K/BxN mice. The onset of arthritis was more rapid in the *Flip*^{f/f}*LysM*^{c/+} mice and the number of synovial tissue neutrophils was increased early in the clinical course, compared with littermate controls. However, peak arthritis was reduced in the *Flip*^{f/f}*LysM*^{c/+} mice, as was the arthritis during the resolution phase. In contrast to expectations, this was not due to a reduction of macrophages. In fact the number of F4/80^{hi} macrophages in the joints of the *Flip*^{f/f}*LysM*^{c/+} mice with arthritis were increased. Further, *Flip* was decreased in the F4/80^{hi} macrophages in the ankles of the *Flip*^{f/f}*LysM*^{c/+} mice, however, the F4/80^{hi} population exhibited an anti-inflammatory phenotype in both groups of mice. These observations suggest that the expression of FLIP, attenuates the development of F4/80^{hi} macrophages under inflammatory conditions and that an increase of F4/80^{hi} macrophages in the *Flip*^{f/f}*LysM*^{c/+} mice results in amelioration of arthritis in the STIA model.

MATERIALS AND METHODS

Mice with *Flip* conditional knockout in myeloid lineage

We generated a genetically modified mouse line with *Flip* floxed (*Flip^{f/f}*) on a C57Bl/6 background as described previously (8). Mice deficient in FLIP in myeloid cells were generated by crossing *Flip^{flox/flox}* (*Flip^{f/f}*) mice with the *LysM^{c/+}* mice (9). Littermates or gender and age matched mice with *Flip^{flox/+}LysM^{c/+}* or *Flip^{f/f}LysM⁺* served as controls. All genotyping was performed by PCR employing genomic DNA extracted from tail biopsies, as described (8). All recombinant DNA and animal procedures were approved by the Office of Research Safety and the Institutional Animal Care and Use Committee of Northwestern University.

K/BxN serum transfer arthritis and analysis

K/BxN mice were generated and anti-GPI serum collected at 8–9 weeks of age as described (10). Arthritis was induced in 7–16 week old *Flip^{f/f}LysM^{c/+}* or littermate control mice employing 150 µl of anti-GPI serum administered intraperitoneally on day 0. The development of arthritis was assessed by measuring hind ankle swelling (thickness, mm) using a caliper and grading the clinical index of all 4 paws/ankles on a scale of 0–3 (maximum = 12) according to previous publications (11, 12). Arthritis was evaluated between days 0 to 14 post-induction. Synovial macrophages were isolated from the ankles before or at day 9 of anti-GPI serum induced arthritis. The expression of IL-10, Resistin-like molecule alpha (Relm-α), Arginase (Arg-1), inducible nitric oxide synthases (iNos) and *Flip* was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cytokines in ankles were determined by ELISA (DuoSets, R&D, Minneapolis, MN) following manufacturer's instructions. To do this, ankles were homogenized in PBS containing 1× of a proteinase inhibitor cocktail and supernatants collected by centrifugation as described (11, 13, 14).

Immuno-phenotyping and blood count

Single cell suspensions were prepared from dissected tibias and paws, incubated in 1 mg/ml collagenase in HBSS buffer for 1 hour as described (11, 14, 15). Cell types were determined by immuno-phenotyping and flow cytometric analysis, employing multi-color fluorochrome-conjugated antibodies to CD11b, F4/80, CD64, Ly6C, Ly6G and MHCII (ebioscience or BD Pharmingen). Data were acquired employing BD LSR II flow cytometer and analyzed by FlowJo (TreeStar, Inc.). Synovial macrophage apoptosis was assessed by Annexin V and cell survival by the exclusion of 7-amino-actinomycin (7AAD, BD Pharmingen). The apoptotic cells were identified as Annexin V⁺ and 7AAD[−] and the necrotic cells are 7AAD⁺ examined by flow cytometry (14). Single cell populations were sorted by BD ARIA for genotyping and Western blot analysis. The complete blood counts and differentials were determined by Hemavet 950 from Drew Scientific Inc.

Immunoblot analysis

Immunoblot analysis was performed according to an established protocol (8). The expression of FLIP were recognized by incubation overnight at 4°C with rabbit monoclonal

antibody to mouse FLIP (Cell Signaling Technology), followed by anti-rabbit secondary antibodies conjugated with horseradish peroxidase (GE healthcare). After stripping, the same membrane were blotted with antibody to GAPDH (Sigma-Aldrich) for loading adjustment. The specific proteins were detected employing the Enhanced Chemiluminescent Detection Reagent (Amersham Pharmacia).

***In vitro* differentiation of macrophages from bone marrow cells**

In vitro bone marrow differentiated macrophages were generated as described (14). Nucleated bone marrow cells were isolated from femurs and tibias of *Flip^{f/f}LysM^{c/+}* or littermate control mice. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100U penicillin-streptomycin, 0.1% (v/v) of 2-mercaptoethanol, 10 µg/ml polymyxin B and the recombinant mouse M-CSF (20ng/ml, R&D Systems). All bone marrow cells were cultured in duplicate with or without the addition of recombinant mouse TNFα (20ng/ml, R&D Systems) and harvested after 7 days culture at 37°C in a 5% CO₂ incubator.

Histological analysis

Ankles were harvested at indicated time points for histological analysis. Ankles were fixed in 10% neutral buffered formalin and then incubated in 10% EDTA decalcification buffer for 2 weeks, embedded in paraffin, and then 4 µm sections were stained with hematoxylin and eosin (H&E). H&E ankle sections was scored 0–5 for joint and extra-articular inflammation, granulocyte infiltration, pannus formation, bone erosion and cartilage destruction as previously described (11, 13, 14). Macrophages in ankles were identified by immunohistochemistry staining employing anti-F4/80 or isotype matched control IgG antibodies. The F4/80⁺ mononuclear cells were the average of 3 views of the synovium in a 1000× field for each of the ankle slide. The osteoclasts were identified by the Tartrate-resistant acid phosphatase (TRAP) staining, and the frequency of osteoclasts were scored (0–4)(16). All H&E, immunohistochemistry staining and TRAP staining slides were evaluated by blinded investigators.

Data analysis

All quantitative data are presented as mean ± SEM. Statistical analysis between groups was done with 2-tailed Student's *t* test, or with one-way ANOVA and Tukey post-test for comparison among multiple groups. Samples that failed the normality test were analyzed by the Mann-Whitney rank sum test. Correlations were determined by Pearson's linear correlation. Significance levels were set at 0.05.

RESULTS

***Flip* conditional knock out in myeloid lineage**

Since the *Flip^{f/d}LysM^{c/+}* mice experienced severe failure to thrive with multi-organ neutrophilic infiltrates (8), *Flip^{f/f}LysM^{c/+}* mice were employed to determine the effect of *Flip* deletion in myeloid cells on the effector phase of inflammatory arthritis employing the serum transfer induced arthritis (STIA). In circulating cells of the *Flip^{f/f}LysM^{c/+}* mice, the deleted floxed allele was seen in monocytes but not T or B lymphocytes (Supplemental

Figure 1A). In granulocytes the *Flip* alleles were nearly completely deleted in the *Flip^{f/f}LysM^{c/+}* mice, which contrasts with the complete deletion in the *Flip^{f/d}LysM^{c/+}* mice (Supplemental Figure 1B). Although mild weight reduction (10~15%), shortened survival, and increased peripheral monocytes and granulocytes were noted in *Flip^{f/f}LysM^{c/+}* mice, these features were all less severe than observed in *Flip^{f/d}LysM^{c/+}* mice (Supplemental Figure 1C–E and (8)), and spontaneous arthritis was not observed.

Following the injection of anti-GPI serum, modestly increased joint swelling ($p < 0.001$) at days 2 and 4 post injection was observed in the *Flip^{f/f}LysM^{c/+}* mice, compared with the littermate controls, although the clinical score was insufficiently sensitive to detect this difference (Figure 1A). However the peak arthritis scores were less in the *Flip^{f/f}LysM^{c/+}* mice, with significantly reduced arthritis during the resolution phase, from day 7 through 14 (Figure 1A). To understand the mechanisms responsible for these differences, ankles were harvested over the course of STIA. Histologically, the inflammation was more severe on day 4, but reduced on day 9 (Figure 1B, C) both in and around the ankle joints of the *Flip^{f/f}LysM^{c/+}* mice. Similar differences were observed for neutrophil infiltration, pannus formation and bone erosion (Figure 1C and Supplemental Figure 2). Both IL-1 β and IL-6 were significantly increased, prior to day 9 in *Flip^{f/f}LysM^{c/+}* mice, compared with littermate controls, although there was no difference of any cytokine on day 9 (Figure 1D). In contrast, IL-10 was significantly reduced on day 4, the peak of arthritis in the *Flip^{f/f}LysM^{c/+}* mice, but it was no different from the controls on days 9 (Figure 1D).

Since bone erosion was reduced at days 9 and 14, and since osteoclasts are derived from myeloid precursors within the inflamed joints, osteoclast numbers were examined. On day 9, a marked decrease ($p < 0.01$) of TRAP positive osteoclasts was identified in the joints of the *Flip^{f/f}LysM^{c/+}* mice compared with those of the littermate controls (Figure 2A). RANKL levels were significantly decreased ($p < 0.01$) while osteoprotegerin (OPG) levels were increased ($p < 0.05$) in *Flip^{f/f}LysM^{c/+}* mouse ankles (Figure 2B). These observations suggest that the more rapid reduction of inflammation following STIA in *Flip^{f/f}LysM^{c/+}* mice was associated with reduced bone erosion due to decreased osteoclastogenesis, which was accompanied by decreased RANKL and increased OPG. These observations may be the result of the reduced inflammation and/or reduced osteoclast survival or differentiation due to *Flip* depletion.

Since FLIP is not expressed in neutrophils (Supplemental Figure 3) and *Flip* deletion in myeloid cells did not diminish neutrophil survival (8) consistent with other reports (17), the effect of myeloid *Flip* deletion on macrophages in the inflamed joints was examined. We hypothesized that deletion of *Flip* in myeloid cells would result in reduced synovial tissue macrophages due to increased apoptosis, resulting in diminished arthritis. First we examined joints prior to the onset of arthritis. To our surprise the number of joint macrophages (CD64⁺CD11b⁺F4/80⁺) was not reduced in *Flip^{f/f}LysM^{c/+}* mice under homeostatic conditions (Figure 3). Macrophages differentiated from recently recruited circulating monocytes are CD64⁺CD11b⁺F4/80⁺Ly6C⁺ (18). Synovial tissue resident macrophages under homeostatic conditions (CD64⁺CD11b⁺F4/80⁺Ly6C[−]MHCII[−]) are long lived, irradiation resistant and suppress arthritis (19). Although the total number of macrophages

was not reduced, the percent of tissue resident macrophages was reduced in *Flip^{f/f}LysM^{c/+}* mice prior to arthritis induction (Figure 3).

Next, the presence of macrophages within the inflamed joints at day 9 was examined by immunohistochemistry. To our surprise, F4/80⁺ macrophages were actually increased ($p < 0.01$) at day 9 in the synovial tissue of ankles from *Flip^{f/f}LysM^{c/+}* mice compared with the littermate controls (Figure 4A). To better characterize the role of macrophages in STIA in the *Flip^{f/f}LysM^{c/+}* mice, the phenotype of macrophages in the joints at 9 days post arthritis induction was examined by flow cytometry. The number of CD11b⁺F4/80^{hi} macrophages was increased ($p < 0.05$) in the *Flip^{f/f}LysM^{c/+}* mice, while there was no difference in the CD11b⁺F4/80^{lo} macrophages (Figure 4B). The number of F4/80^{hi} macrophages, as well as the level of expression of F4/80, inversely correlated ($p = 0.005$ – 0.001) with joint swelling on clinical exam (Figure 4C).

In order to determine if there were functional differences between CD11b⁺F4/80^{hi} and CD11b⁺F4/80^{lo} macrophages, these populations were isolated by flow cytometry from the ankles 9 days after arthritis induction (Figure 5). In the control mice, by qRT-PCR, the expression of IL-10 and Relm- α were increased ($p < 0.001$ – 0.01) in the F4/80^{hi} population while the expression of iNos was increased ($p < 0.01$) in those that were F4/80^{lo} (Figure 5A, white bars). Arg-1 was not different between the populations. Similar differences were noted between the F4/80^{hi} and F4/80^{lo} populations in the *Flip^{f/f}LysM^{c/+}* mice (Figure 5A, dark bars). In prior studies (8), under homeostatic conditions, any macrophages isolated from *Flip^{f/d}LysM^{c/+}* mice still expressed *Flip*. To our surprise *Flip*, although present, was significantly ($p < 0.05$) reduced in the F4/80^{hi} macrophages from the *Flip^{f/f}LysM^{c/+}* mice compared with the littermate controls (Figure 5B). No significant difference of *Flip* was observed in the F4/80^{lo} macrophages between the *Flip^{f/f}LysM^{c/+}* and control mice (Figure 5B). To understand where the change in the expression of *Flip* occurred, monocytes from *Flip^{f/f}LysM^{c/+}* and control mice were isolated from bone marrow, which are the immediate precursors of circulating monocytes, by flow cytometry. Compared with blood T lymphocytes, FLIP was variably expressed in the bone marrow derived monocytes from both *Flip^{f/f}LysM^{c/+}* and control mice (Supplemental Figure 3). Since the monocyte derived macrophages observed in STIA derive primarily from circulating monocytes which express FLIP in both the *Flip^{f/f}LysM^{c/+}* and control mice, it is likely that the reduction of *Flip* in *Flip^{f/f}LysM^{c/+}* F4/80^{hi} macrophages occurs following ingress to the inflammatory environment. Since circulating monocytes demonstrate a deletion of one *Flip* allele, we speculate that after arriving in the inflamed joints that increased expression of Cre driven by the LysM promoter may result in deletion of the second allele.

Ankles were examined to determine if reduction of *Flip* resulted in increased macrophage apoptosis during STIA. There was no increased apoptosis or necrosis of the macrophages in the ankles of the *Flip^{f/f}LysM^{c/+}* mice compared with the controls at day 9 of STIA, consistent with the ability of the F4/80^{hi} macrophages in the *Flip^{f/f}LysM^{c/+}* mice to survive despite the reduction of *Flip* (Figure 6A). Next we used bone marrow derived cells from control or *Flip^{f/f}LysM^{c/+}* mice which were *in vitro* differentiated into macrophages in the presence of M-CSF, with or without TNF α , to simulate inflammatory conditions. After 7 days, as expected in the presence of reduced *Flip*, the number of macrophages from the

Flip^{f/f}LysM^{c/+} were decreased (Figure 6B). However, in contrast to culture in the absence of TNF α , macrophages expressing reduced levels of FLIP survived (Figure 6B). These observations suggest that in absence of inflammation macrophages expressing FLIP preferentially survived and expanded, while under inflammatory conditions, macrophages with reduced FLIP are capable of survival.

DISCUSSION

Since FLIP is highly expressed in RA synovial tissue macrophages, protecting them against Fas-FasL mediated apoptosis (5, 6), we reasoned that suppression of FLIP in macrophages within the inflamed joint would be therapeutically beneficial by reducing the number of macrophages. The effective deletion of *Flip* in myeloid cells (*Flip^{f/d}LysM^{c/+}* mice) resulted in a marked reduction of macrophages and a dramatic phenotype, including early mortality due to multi-organ neutrophil infiltrates (8). Therefore we employed *Flip^{f/f}LysM^{c/+}* mice which exhibited less severe phenotypic changes. To our surprise, the onset of arthritis in the *Flip^{f/f}LysM^{c/+}* mice was more abrupt, while peak arthritis was reduced and the resolution phase was more complete. Unexpectedly under homeostatic conditions, before the induction of STIA, there was no reduction of total synovial tissue macrophages in the *Flip^{f/f}LysM^{c/+}* mice, although tissue resident, arthritis-suppressive (19), macrophages were reduced. Further, during the resolution phase of the arthritis an increase of F4/80^{hi} macrophages was found in the joints of the *Flip^{f/f}LysM^{c/+}* mice which inversely correlated with disease activity. This population exhibited a reduction of *Flip* and an M2-like phenotype, which may have been responsible for the enhanced resolution of STIA in these mice. However, M1/M2 polarization of bone marrow differentiated macrophages did not affect *Flip* (data not shown). Since IL-10 was not increased at day 9 in the joints of the *Flip^{f/f}LysM^{c/+}* mice, it is possible that the change from day 4 was important although it is likely that other mechanisms are also responsible. These observations cannot be generalized since other forms of arthritis were not examined.

Recent studies have demonstrated in many tissues that under homeostatic conditions tissue resident macrophages are F4/80^{hi} (18, 20–22). Microglia are MHCII[−] while those in other tissues express MHCII at variable levels (18). By fate mapping, there is agreement that microglia originate from the yolk sac, and that those in the intestine are derived from fetal hematopoietic stem cells, although there is controversy as to the origin of other tissue resident macrophages including those in the lung, liver and spleen (18, 21). All agree that tissue resident macrophages are long lived, self-populating and radiation resistant. While fate mapping has not been employed for synovial tissue, under homeostatic conditions, CD11b⁺CD64⁺Ly6C[−]MHCII[−] synovial tissue macrophages are long lived and radiation resistant, suggesting that they are indeed tissue resident macrophages (19). Under homeostatic conditions, a portion of these cells are F4/80^{hi} (Figure 3A) in control and *Flip^{f/f}LysM^{c/+}* mice. It remains to be determined, under conditions in which inflammation resolves, to what degree the F4/80^{hi} macrophages derived from circulating monocytes take on the characteristics and function of bona fide tissue resident macrophages.

A previous study of STIA demonstrated that Ly6C[−] monocytes were necessary and sufficient for the initiation and propagation of the disease, although neutrophils and Ly6C⁺

monocytes contributed to disease pathogenesis (19). In the *Flip^{f/f}LysM^{c/+}* mice, under homeostatic conditions, Ly6C⁺ classical monocytes are increased. Non-classical, Ly6C⁻ monocytes are critical for the initiation of STIA, and CCR2⁻ mice develop STIA comparably to the wild type controls (19). Therefore, it seems unlikely that the increase of Ly6C⁺ monocytes, which are also CCR2⁺, observed in the *Flip^{f/f}LysM^{c/+}* mice prior to arthritis initiation (Supplemental Figure 1), contributed to the increased arthritis observed on days 2 and 4. It is more likely that the more severe arthritis observed early in the clinical course of the *Flip^{f/f}LysM^{c/+}* mice was due to the reduction of the tissue resident macrophages, since the deletion of MHCII⁻ tissue resident macrophages prior to disease onset resulted in increased STIA (19). Additionally the *Flip^{f/f}LysM^{c/+}* mice exhibit increased circulating neutrophils which may also have contributed to the increased arthritis early in the clinical course.

During active inflammation in STIA, monocytes recruited locally become polarized toward M1-like pro-inflammatory macrophages, however during the resolution phase the macrophages transition to a more M2-like phenotype (19). At day 9, the peak of arthritis in the control mice, and a time when the *Flip^{f/f}LysM^{c/+}* mice were clinically improving, an increased subset of F4/80^{hi} macrophages was identified in the *Flip^{f/f}LysM^{c/+}* mice. This population of macrophages was inversely correlated with the severity of the arthritis at the time of sacrifice. Further when cells were isolated from inflamed joints, the F4/80^{hi} population expressed an increased M2-like phenotype, compared to the F4/80^{lo} population of macrophages. These observations support the importance of macrophage repolarization as a therapeutic approach to inflammatory arthritis.

We initially intended to target *Flip* in myeloid cells as a means to therapeutically reduce synovial tissue macrophages by inducing apoptosis. Our earlier studies with human monocytes and macrophages demonstrated that FLIP was weakly expressed in monocytes, but highly expressed during monocyte to macrophage differentiation (5). Even though lysozyme M is expressed in monocytes (23), and the deleted *Flip* allele was seen in monocytes, the monocytes from *Flip^{f/f}LysM^{c/+}* and control mice variably expressed FLIP. Consistent with these observations, there was no difference in the expression of *Flip* in the F4/80^{lo} population in the *Flip^{f/f}LysM^{c/+}* mice, compared with the controls. In contrast, the F4/80^{hi} macrophages demonstrated a significant reduction of *Flip* in the *Flip^{f/f}LysM^{c/+}* mice. This suggests that under inflammatory conditions, the requirement of FLIP for survival in macrophages may be circumvented. Supporting this interpretation there was no increase of apoptosis or necrosis of the F4/80^{hi} population in *Flip^{f/f}LysM^{c/+}* mice. Further, under inflammatory conditions bone marrow derived macrophages from *Flip^{f/f}LysM^{c/+}* mice survived in the presence of reduced FLIP. These observations may be related to an increase of other anti-apoptotic molecules such as Mcl-1 or A20 which may also contribute to macrophage survival (24–27) and which are increased under inflammatory conditions.

A reduction of FLIP in surviving macrophages may have modulated intracellular signals increasing the differentiation toward F4/80^{hi}, M2-like macrophages. Although caspase cleaved products of FLIP have been shown to activate NF- κ B (28, 29), unprocessed FLIP may suppress NF- κ B activation induced by LPS or Fas signaling (30, 31). Further the interaction of FLIP with the Fas-DISC while protecting against apoptosis recruits MyD88,

suppressing TLR4 signaling (32), suggesting that the effects of FLIP may be context specific. FLIP also binds to MKK7 inhibiting JNK signaling (33), and it participates in activation of inflammasomes, and reduction of FLIP may be suppressive (34). FLIP is also capable of promoting Wnt signaling through reduction of β -catenin ubiquitination (35), which may modulate macrophage activation (36). FLIP may also suppress p38 activation in dendritic cells (37). Our data are consistent with the interpretation that under inflammatory conditions macrophages may survive despite the reduction of FLIP. Other studies suggest that not only inflammation, but also stress from hypoxia and energy deprivations, may protect cells from apoptosis (38). Therefore in macrophages capable of survival despite the reduction of FLIP under inflammatory conditions, the reduction of FLIP may promote an increase in the number of F4/80^{hi} macrophages with an M2-like phenotype, potentially through alteration of intracellular signaling. Alternatively, in the control mice, the high expression FLIP may suppress this transition to F4/80^{hi}. Together these observations suggest that under inflammatory conditions the reduction of FLIP in macrophages ameliorates STIA, not by reduction of macrophages but by permitting an increase of F4/80^{hi} macrophages with an M2-like phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Gierut A, Perlman H, Pope RM. Innate immunity and rheumatoid arthritis. *Rheum Dis Clin North Am.* 2010; 36:271–96. [PubMed: 20510234]
2. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med.* 2011; 365:2205–19. [PubMed: 22150039]
3. Mulherin D, Fitzgerald O, Bresnihan B. Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. *Arthritis Rheum.* 1996; 39:115–24. [PubMed: 8546720]
4. Wijbrandts CA, Vergunst CE, Haringman JJ, Gerlag DM, Smeets TJ, Tak PP. Absence of changes in the number of synovial sublining macrophages after ineffective treatment for rheumatoid arthritis: Implications for use of synovial sublining macrophages as a biomarker. *Arthritis Rheum.* 2007; 56:3869–71. [PubMed: 17968928]
5. Perlman H, Pagliari LJ, Georganas C, Mano T, Walsh K, Pope RM. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. *J Exp Med.* 1999; 190:1679–88. [PubMed: 10587358]
6. Perlman H, Pagliari LJ, Liu H, Koch AE, Haines GK 3rd, Pope RM. Rheumatoid arthritis synovial macrophages express the Fas-associated death domain-like interleukin-1 β -converting enzyme-inhibitory protein and are refractory to Fas-mediated apoptosis. *Arthritis Rheum.* 2001; 44:21–30. [PubMed: 11212162]
7. Okada Y, Wu D, Trynka G, Raj T, Chikashi T, Ikari K, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature.* 2014; 506:376–81. [PubMed: 24390342]
8. Huang QQ, Perlman H, Huang Z, Birkett R, Kan L, Agrawal H, et al. FLIP: a novel regulator of macrophage differentiation and granulocyte homeostasis. *Blood.* 2010; 116:4968–77. [PubMed: 20724542]

9. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 1999; 8:265–77. [PubMed: 10621974]
10. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. *Cell.* 1996; 87:811–22. [PubMed: 8945509]
11. Huang QQ, Birkett R, Koessler RE, Cuda CM, Haines G 3rd, Jin JP, et al. Fas Signaling in Macrophages Promotes Chronicity in K/BxN Serum-Induced Arthritis. *Arthritis Rheumatol.* 2014; 66:68–77. [PubMed: 24431281]
12. Scatizzi JC, Hutcheson J, Bickel E, Woods JM, Klosowska K, Moore TL, et al. p21Cip1 is required for the development of monocytes and their response to serum transfer-induced arthritis. *Am J Pathol.* 2006; 168:1531–41. [PubMed: 16651620]
13. Huang QQ, Koessler RE, Birkett R, Dorfleitner A, Perlman H, Kenneth Haines G 3rd, et al. Gp96 perpetuates the persistent inflammation of rheumatoid arthritis. *Arthritis Rheum.* 2012
14. Huang QQ, Perlman H, Birkett R, Doyle R, Fang D, Haines GK, et al. CD11c-mediated deletion of Flip promotes autoreactivity and inflammatory arthritis. *Nat Commun.* 2015; 6:7086. [PubMed: 25963626]
15. Zhang Q, Lu Y, Proulx ST, Guo R, Yao Z, Schwarz EM, et al. Increased lymphangiogenesis in joints of mice with inflammatory arthritis. *Arthritis Res Ther.* 2007; 9:R118. [PubMed: 17997858]
16. Zhang Q, Badell IR, Schwarz EM, Boulukos KE, Yao Z, Boyce BF, et al. Tumor necrosis factor prevents alendronate-induced osteoclast apoptosis in vivo by stimulating Bcl-xL expression through Ets-2. *Arthritis Rheum.* 2005; 52:2708–18. [PubMed: 16142752]
17. Haverkamp JM, Smith AM, Weinlich R, Dillon CP, Qualls JE, Neale G, et al. Myeloid-derived suppressor activity is mediated by monocytic lineages maintained by continuous inhibition of extrinsic and intrinsic death pathways. *Immunity.* 2014; 41:947–59. [PubMed: 25500368]
18. Sheng J, Ruedl C, Karjalainen K. Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells. *Immunity.* 2015; 43:382–93. [PubMed: 26287683]
19. Misharin AV, Cuda CM, Saber R, Turner JD, Gierut AK, Haines GK 3rd, et al. Nonclassical Ly6C(–) monocytes drive the development of inflammatory arthritis in mice. *Cell Rep.* 2014; 9:591–604. [PubMed: 25373902]
20. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* 2013; 38:79–91. [PubMed: 23273845]
21. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature.* 2015; 518:547–51. [PubMed: 25470051]
22. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol.* 2014; 14:392–404. [PubMed: 24854589]
23. Eierman DF, Johnson CE, Haskill JS. Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates. *J Immunol.* 1989; 142:1970–6. [PubMed: 2784145]
24. Zhou P, Qian L, Bieszczad CK, Noelle R, Binder M, Levy NB, et al. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. *Blood.* 1998; 92:3226–39. [PubMed: 9787159]
25. Tran TM, Temkin V, Shi B, Pagliari L, Daniel S, Ferran C, et al. TNFalpha-induced macrophage death via caspase-dependent and independent pathways. *Apoptosis.* 2009; 14:320–32. [PubMed: 19152111]
26. Liu H, Perlman H, Pagliari LJ, Pope RM. Constitutively activated Akt-1 is vital for the survival of human monocyte-differentiated macrophages. Role of Mcl-1, independent of nuclear factor (NF)-kappaB, Bad, or caspase activation. *J Exp Med.* 2001; 194:113–26. [PubMed: 11457886]
27. Li HL, Wang AB, Zhang R, Wei YS, Chen HZ, She ZG, et al. A20 inhibits oxidized low-density lipoprotein-induced apoptosis through negative Fas/Fas ligand-dependent activation of caspase-8 and mitochondrial pathways in murine RAW264.7 macrophages. *J Cell Physiol.* 2006; 208:307–18. [PubMed: 16646083]
28. Golks A, Brenner D, Krammer PH, Lavrik IN. The c-FLIP-NH2 terminus (p22-FLIP) induces NF-kappaB activation. *J Exp Med.* 2006; 203:1295–305. [PubMed: 16682493]

29. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmeler M, et al. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr Biol.* 2000; 10:640–8. [PubMed: 10837247]
30. Bannerman DD, Eiting KT, Winn RK, Harlan JM. FLICE-like inhibitory protein (FLIP) protects against apoptosis and suppresses NF-kappaB activation induced by bacterial lipopolysaccharide. *Am J Pathol.* 2004; 165:1423–31. [PubMed: 15466406]
31. Kreuz S, Siegmund D, Rumpf JJ, Samel D, Leverkus M, Janssen O, et al. NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *J Cell Biol.* 2004; 166:369–80. [PubMed: 15289496]
32. Ma Y, Liu H, Tu-Rapp H, Thiesen HJ, Ibrahim SM, Cole SM, et al. Fas ligation on macrophages enhances IL-1R1-Toll-like receptor 4 signaling and promotes chronic inflammation. *Nat Immunol.* 2004; 5:380–7. [PubMed: 15004557]
33. Nakajima A, Komazawa-Sakon S, Takekawa M, Sasazuki T, Yeh WC, Yagita H, et al. An antiapoptotic protein, c-FLIPL, directly binds to MKK7 and inhibits the JNK pathway. *EMBO J.* 2006; 25:5549–59. [PubMed: 17110930]
34. Wu YH, Kuo WC, Wu YJ, Yang KT, Chen ST, Jiang ST, et al. Participation of c-FLIP in NLRP3 and AIM2 inflammasome activation. *Cell Death Differ.* 2014; 21:451–61. [PubMed: 24270411]
35. Naito M, Katayama R, Ishioka T, Suga A, Takubo K, Nanjo M, et al. Cellular FLIP inhibits beta-catenin ubiquitylation and enhances Wnt signaling. *Mol Cell Biol.* 2004; 24:8418–27. [PubMed: 15367663]
36. Schaale K, Neumann J, Schneider D, Ehlers S, Reiling N. Wnt signaling in macrophages: augmenting and inhibiting mycobacteria-induced inflammatory responses. *Eur J Cell Biol.* 2011; 90:553–9. [PubMed: 21185106]
37. Wu YJ, Wu YH, Mo ST, Hsiao HW, He YW, Lai MZ. Cellular FLIP Inhibits Myeloid Cell Activation by Suppressing Selective Innate Signaling. *J Immunol.* 2015; 195:2612–23. [PubMed: 26238491]
38. Milisav I, Poljsak B, Ribaric S. Reduced risk of apoptosis: mechanisms of stress responses. *Apoptosis.* 2017; 22:265–83. [PubMed: 27807740]

Figure 1. *Flip^{f/f}LysM^{c/+}* KO mice exhibit enhanced early but attenuated late phase of STIA (A). The course of the arthritis was documented as the clinical activity score (left panel) and the ankle swelling (ankle thickness in mm for hind ankles, right panel). Data were combined from 5 independent experiments, employing 40 *Flip^{f/f}LysM^{c/+}* or control mice. After sacrificing to harvest samples at days 2 and 4, 18–19 remaining mice were scored on day 9, and 7 mice on day 14 post arthritis induction. (B). Representative H&E histology imaging from the ankles of control and *Flip^{f/f}LysM^{c/+}* mice harvested at day 4 and day 9. (C). The histological score of ankle joints for joint and extra-articular inflammation, PMN infiltration, pannus formation and bone erosion over the course of STIA (n= 7–16 ankles/group). (D). IL-1β, IL-6 and IL-10 in ankles determined by ELISA. Data are presented as the mean ±1 SE. Significance between the groups was determined by unpaired 2-sided *t*-test. * represents *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001 between the indicated groups.

Figure 2. Attenuated STIA is associated with decreased osteoclastogenesis

(A). Representative TRAP-stained ankle sections from mice that were sacrificed on day 9 post arthritis induction. Numerous TRAP+ osteoclasts (indicated by the arrows) were detected in the ankle joints of control mice (left panel). The frequency of osteoclasts were scored (0–4) and analyzed (right panel, n=7 *Flipf/f LysM^{c/+}* mice and 8 controls). (B). The expression levels of RANKL and OPG in ankles at day 9 post arthritis induction mice was quantified by ELISA (n=17 *Flipf/f LysM^{c/+}* and 20 control ankles). Data are presented as the mean $\pm$ 1 SE. Significance was analyzed by unpaired 2-sided *t*-test for score and OPG analysis, and by Mann-Whitney test for RANKL. *represents $p < 0.05$ and ** $p < 0.01$.

Figure 3. *Flipf/fLysM^{c/+}* ankles exhibit reduced synovial tissue resident macrophages
(A). A representative flow cytometric analysis of ankle macrophages under steady state conditions presenting the gating strategy. After exclusion of doublets and debris, and gating out granulocytes, dendritic cells and B cells, synovial macrophages were identified as CD64⁺CD11b⁺F4/80⁺. After removing Ly6C⁺ and MHCII⁺ cells, synovial tissue resident macrophages are identified as CD64⁺CD11b⁺F4/80⁺Ly6C⁻MHCII⁻. **(B).** Analysis of the total synovial macrophages and the percent of synovial tissue resident macrophages from ankles of *Flipf/fLysM^{c/+}* mice and the control littermates prior to induction of STIA. (n = 13 for *Flipf/fLysM^{c/+}* mice and 15 for the controls). Data are presented as the mean ± 1 SE. Significance was analyzed by unpaired 2-sided *t*-test. * represents *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001 between the indicated groups

Figure 4. *Flipf/f LysM^{c/+}* mice exhibit increased synovial F4/80⁺ macrophages by immunohistochemistry and increased F4/80^{hi} synovial macrophages in *Flipf/f LysM^{c/+}* mice which correlate with reduced STIA

(A). Immunohistochemistry employing anti-F4/80 was performed to identify the distribution of macrophages in the ankles of mice day 9 post arthritis induction. The left panels are representative anti-F4/80-staining of ankles from control and *Flipf/f LysM^{c/+}* mice. The F4/80⁺ cells are brown. Synovial tissue F4/80⁺ cells were counted and presented as the average from 3 fields for each ankle ($n = 7$ *Flipf/f LysM^{c/+}* and 8 controls) present at right.

(B). Representative flow cytometry images of ankles from control and *Flipf/f LysM^{c/+}* mice 9

days post arthritis induction. After gating out granulocytes and CD11b⁻ cells, macrophages were identified as CD64⁺CD11b⁺, and further as F4/80^{hi} and F4/80^{lo}. (C). The correlations between arthritis severity (ankle thickness, mm) and the number of F4/80^{hi} macrophages and the mean fluorescence intensity (MFI) of F4/80 expression are presented (n = 7 control and 8 *Fltp^{fl/fl}LysM^{c/+}* mice). Significance was determined by unpaired 2-sided *t*-test (A,B), * represents *p* < 0.05 and ** *p* < 0.01 between the indicated groups. Pearson linear correlation was performed to determine correlations (C).

Figure 5. During STIA, F4/80^{hi} synovial macrophages exhibit an M2-like phenotype and reduced *Flip* expression

Macrophages were purified by flow cytometry from the ankles of control and *Flip^{f/f}LysM^{C/+}* mice 9 days post STIA induction. Macrophages were gated as CD11b⁺, CD64⁺ F4/80^{hi} and F4/80^{lo} as in Figure 4B. (A). The expression of IL-10, Resistin-like molecule alpha (Relm-α), Arginase (Arg-1) and inducible nitric oxide synthases (iNos) determined by qRT-PCR, presented as the fold of expression normalized to the F4/80^{hi} cells of the control mouse group. Data were compared between the F4/80^{hi} and F4/80^{lo} populations of the control or *Flip^{f/f}LysM^{C/+}* mice for each molecule (n=6 control and 3 *Flip^{f/f}LysM^{C/+}* mice). Significance was determined by unpaired 2-sided *t*-test (B). The expression of *Flip* in the same samples was also determined by qRT-PCR, presented as fold of expression normalized to control F4/80^{hi} cells. Analysis was performed by one-way ANOVA, and Tukey post-test. * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between the indicated groups

Figure 6. No difference in the apoptosis or necrosis of synovial macrophages at day 9 of STIA between *Flip^{f/f}LysM^{c/+}* and control mice

(A). Apoptotic cells were defined as Annexin V⁺ and 7AAD⁻ and necrotic cells as Annexin V⁺ and 7AAD⁺ in CD64⁺CD11b⁺F4/80^{hi} and F4/80^{lo} synovial macrophages, harvested at 9 days post arthritis induction. Representative flow images for apoptosis defined in the F4/80^{hi} population of control and *Flip^{f/f}LysM^{c/+}* mice are presented in the panels on the left and the percent of apoptotic or necrotic cells in the panels on the right. This analysis was from 5 control and 7 *Flip^{f/f}LysM^{c/+}* mice. Significance was determined by unpaired 2-sided *t*-test.

(B). Bone marrow cells from *Flip^{f/f}LysM^{c/+}* or littermate control mice differentiated for 7 days in the presence of M-CSF, with or without TNF α . FLIP expression were determined by Western blot. The blot presented is representative of 2 independent experiments.