M-CSF-Stimulated CD11b⁺ Myeloid Cells Induce Alopecia Areata in C3H/HeJ Mice

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Abstract

Alopecia areata (AA) is a T cell-mediated autoimmune skin disease with clinical features of hair loss and skin inflammation. It is unclear whether other immune cells except T lymphocytes are also involved in the development of AA. Here, our results reveal that dermal injection of either CD11b⁺ myeloid cells isolated from AA-affected skin or non-AA splenocyte-derived CD11b⁺ cells treated with macrophage colony-stimulating factor (M-CSF) induces AA in C3H/HeJ mice. The functional similarity of these cells in induction of AA seems to be due to a higher expression of M-CSF in AA affected skin. To explore the mechanism by which dermal injection of CD11b⁺ cells induce AA, we co-culture either AA derived skin cells or M-CSF-stimulated CD11b⁺ cells with naïve splenocytes. The results of splenocyte proliferation assay and immunoglobulin release in conditioned medium show a significant increase of splenocyte proliferation and IgG level in conditioned medium under both conditions as compared to controls. Most activated splenocytes induced by M-CSF-stimulated myeloid cells are B lymphocytes. B cell activation are further confirmed in AA-affected skin and skin draining lymph nodes of AA mice. In conclusion, in this study, we have provided evidence that M-CSF stimulated CD11b⁺ cells are able to induce AA in C3H/HeJ mice through a possible mechanism by activating B lymphocytes. This finding may provide insight for understanding the pathogenesis of AA.

Introduction

Alopecia areata (AA) is one of the most common autoimmune skin diseases in men and women with a lifetime incidence risk of approximately 2.1%¹. Hair loss in AA is often accompanied by local skin inflammation². Although T cell-mediated immune activation has been speculated to drive skin inflammation and hair loss in AA^{3,4,5,6}, clear-cut experimental evidence for the role of T cells in initiation and development of AA is scarce. Whether other immune cells are involved in T cell activation and how T cells remain constitutively active in skin lesions of AA are still largely unexplored.

Diverse immune cell populations have been described in many autoimmune diseases. For example, the immune cell subsets found in the inflamed central nervous system of autoimmune multiple sclerosis include macrophages, monocytes, dendritic cells, B cells, T cells and natural killer cells^{7,8}. In AA lesions, it has been reported that plasmacytoid dendritic cells⁹, macrophages¹⁰, Langerhans cells¹¹, natural killer cells¹² and mast cells¹³ were present in addition to T lymphocytes, suggesting a possible cross-talk between these immune cell populations and T lymphocytes or a similar role of these immune cells with T lymphocytes in AA development. This notion was further supported by studies showing that CD8⁺ effector T cells can promote macrophage recruitment¹⁴, CD4⁺ T cells can instruct myeloid cells to produce inflammasome-independent IL-1 β and cause autoimmunity¹⁵, and memory B cells can activate brain-homing autoreactive CD4⁺ T cells in multiple sclerosis¹⁶.

Myeloid cells including monocytes, macrophages, dendritic cells and granulocytes, are largely responsible for innate defense against various pathogens. Some of the myeloid cells that are antigen presenting cells

also participate in adaptive immunity. It is well-known that myeloid cells contribute to chronic inflammation in impaired healing wounds of patients with diabetes ¹⁷, drive chronicity in rheumatic arthritis ¹⁸, and are therapeutic targets to decrease neuroinflammation after a stroke ¹⁹. In the case of AA, studies have found infiltration of plasmacytoid dendritic cells, macrophages and Langerhans cells along with activated T lymphocytes in anagen follicles ^{10, 20, 21, 22}. All these studies suggest that myeloid cells may play a key role in AA development. However, the precise identity of myeloid cell subsets and the mechanism through which they act in induction of AA have not been explored. In the current study, we took advantage of an AA mouse model previously established by our group ²³ to investigate the role of AA derived CD11b⁺ cells in AA induction and action of other immune cells.

Methods

Mice

Eight weeks-old female C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experimental procedures were approved by the University of British Columbia Animal Care Committee. The methods were carried out in accordance with the approved guidelines. All mice were euthanized by carbon dioxide gas after anesthesia by isoflurane inhalation, according to the recommendation by Animal Care Committee, at indicated time points. Tissues were collected for histological and FACS analyses.

Antibodies

All primary and secondary antibodies used in this study were commercially available: M-CSF antibody (R & D system, Minneapolis, MN, Ab-416-NA); CD3 antibody (Abcam, Ab828); CD3e-PE antibody (Invitrogen, 12-0031-85); CK14 antibody (Abcam, Cambridge, MA, Ab7800); Ki67 antibody (Invitrogen, Burlington, ON, Canada, 14-5698-82); P63 antibody (Invitrogen, PA5-36069); CD11b antibody from two source (Invitrogen, 14-0112-8; Abcam, Ab133357); CD11b-PE antibody (Invitrogen, 12-0031-85); CD19 antibody (Invitrogen, 14-0194-82); CD19-eFluor 660 antibody (Invitrogen, 50-0193-80); Anti-mouse IgG HRP-conjugated secondary antibody (Bio-rad, Mississauga, ON, Canada, 172-1011); Anti-mouse IgG alexa Fluor 568 secondary antibody (Invitrogen, A11004); Anti-mouse IgG alexa Fluor 488 secondary antibody (Invitrogen, A11029); Anti-rat IgG alexa Fluor 488 secondary antibody (Invitrogen, A11006); Anti-rat IgG Rhodamine red secondary antibody (Jackson ImmunoRes Lab, West Grove, PA, 112-295-003); Anti-rabbit IgG Rhodamine red secondary antibody (Jackson ImmunoRes Lab, 111-295-003); Anti-goat IgG alexa Fluor 568 secondary antibody (Millipore-Sigma, Oakville, ON, Canada, A5420).

H & E staining

For histological examination, skin tissues were fixed with 10% formalin, embedded in paraffin, sectioned, and stained with H & E solution (Millipore-Sigma). Photomicroscope images were captured with a Leica microscope using a DS-Ri1 camera and NIS-Elements software (Nikon, Mississauga, ON, Canada).

Cell isolation from mouse skin

To isolate skin cell mixture from mouse skin, about 80% body surface area of skin was harvested and washed three times with phosphate buffer saline (PBS) containing three-fold antibiotic-antimycotic (Invitrogen). Skin was minced into small pieces (about 2-3 mm × 2-3 mm) and digested by incubation with 1 mg/mL collagenase IV (Millipore-Sigma) in PBS for one hour with shaking (250 rpm). The collagenase was then neutralized by adding an equal volume of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). Cells were filtered through a 70 µm cell strainer (Invitrogen) and washed twice with DMEM containing 10% FBS. Cells were cultured in DMEM with 2% FBS for further experiments.

Immune cell purification from mouse splenocytes.

CD11b⁺ myeloid cells from mouse splenocytes, AA-affected skin and cultured skin cell mixture were isolated using Easy SepTM mouse CD11b positive selection kit II according to the protocol (Stem Cell Technologies, Vancouver, BC, Canada).

Immunofluorescent staining in skin tissue and cultured cells

Cell and skin samples were fixed with 10% formalin. For tissue samples, 5 µm sections were cut and used for immunofluorescent staining. Following deparaffinization and antigen retrieval, skin sections were incubated overnight with primary antibody after blocking non-specific binding with blocking solution containing 5% bovine serum albumin for one hour. For cell staining, cells were directly blocked with blocking solution and incubated with primary antibody overnight without antigen retrieval. After washing three times with PBS or PBS-T (0.1% Triton X-100 in PBS) at room temperature, sections or cells were incubated with secondary, fluorescein-conjugated antibodies for another one hour. After washing three times, cells (or sections) were stained with 1.5% Vectashield mounting medium containing DAPI (Vector Laboratories, Burlington, ON, Canada). Images were captured using a Zeiss Axioplan 2 fluorescence microscope and AxioVision image analysis software (Carl Zeiss Canada Ltd, Toronto, ON, Canada).

Western blot

Total proteins were extracted by cell lysis buffer [50 mM Tris-HCl, 5 mM EDTA, 1mM EGTA, 1% TritonX-100, 0.5% Igepal CA-630 and protease inhibitor cocktail (Millipore-Sigma)]. To extract proteins from skin tissue, skin was minced in small pieces and sonicated for one minute with 50% power. The concentration of proteins was measured by BCA protein assay (Pierce, Invitrogen Life Technologies). Proteins were denatured and reduced before being fractionated by 10% SDS-PAGE. Proteins were then transferred onto a PVDF membrane (Millipore-Sigma) and blocked with PBS-T (0.1% Tween 20) containing 5% skim milk for one hour before overnight incubation with primary antibody. The membrane was then washed five times with PBS-T and incubated for another one hour with the secondary antibody conjugated to horseradish peroxidase (HRP). After being washed five times with PBS-T, the specific signal was visualized by an enhanced chemiluminescence (ECL) assay kit (Santa Cruz Biotechnologies).

Generation of M-CSF-stimulated CD11b⁺ myeloid cells from mouse splenocytes

Spleens from C3H/HeJ mice were kept in DMEM, minced mechanically with a 10 mL syringe plug, and passed through a 40 µm cell strainer. Red blood cells were lysed by incubation with ammonium chloride lysis buffer for 5 min. Cells were washed three times with DMEM containing 2% FBS. Splenocytes, at a cell density of $0.5-1 \times 10^6/\text{mL}$ were cultured in DMEM containing 2% FBS and 5 ng/mL M-CSF (Invitrogen) at 37°C and 5% CO₂. After 3 days, cells were washed three times with PBS to remove suspended lymphocytes and were continued to be cultured in DMEM containing 2% FBS and 5 ng/mL M-CSF until use. The medium was changed daily and cells at passage 1-2 were used for this study.

Co-culture experiments

M-CSF stimulated CD11b⁺ myeloid cells derived from the splenocytes of C3H/HeJ mice were sub-cultured in 24-well plates at a cell density of $1 \times 10^5/\text{well}$ overnight in 0.5 mL of minimum essential media (MEM, Thermo Fisher Scientific) containing 2% FBS and 5 ng/ml of M-CSF. Freshly isolated splenocytes from healthy mice of the same strain, with or without prior labelling with CFSE (Invitrogen), at a cell density of $2 \times 10^6/\text{well}$ were added on top of the cultured myeloid cells. After 4 days of co-culture, the suspended splenocytes were harvested for FACS analysis. In some of the experiments, 50% of the filtered conditioned medium was collected from the M-CSF-stimulated splenocyte-derived myeloid cells, passed through a 0.22 µm filter, and 50% fresh MEM containing 2% FBS was directly added to the splenocytes for culture. In other experiments, we separated the co-cultured myeloid cells from the splenocytes with a Trans-well insert with pores of 0.4 µm (Corning Costar, NY). To measure IgG level in cell culture medium, mouse splenocytes were cultured in 50% conditioned medium from M-CSF-treated myeloid cells and 50% fresh medium for 5 days.

To co-culture skin adherent cells with CFSE-labeled splenocytes, a similar procedure was performed. To remove myeloid cells from cultured skin cell mixture, 0.12 ml of clodronate liposome per ml medium was added to treat cells for 48 hrs. After washing, the same number of cells from different group was further sub-cultured and co-cultured with CFSE-labeled splenocytes for 4 days.

FACS analyses

Cells in live were incubated with primary antibodies conjugated to fluorescein for one hour at room temperature. After washing three times with PBS, stained cells were analyzed by BD Accuri C6 cytometer and C6 analysis software (BD Biosciences, Mississauga, ON, Canada). For CFSE proliferation assay, splenocytes were labeled with CFSE (Invitrogen) prior to co-culture. After co-culture, non-adherent lymphocytes were collected and washed three times. Cell proliferation was detected by FACS analysis.

Dermal injection of myeloid cells to C3H/HeJ mice

Myeloid cells in 100 µL DMEM containing 2% FBS were dermally injected in one spot of dorsal skin under general anesthesia. Mice were followed for the indicated time period. Mice with hair loss were diagnosed as having AA-like phenotype.

Statistical Analyses

All data were presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed with GraphPad Prism 8.0 software. *P* values were calculated using two-tailed unpaired student's *t* test. The Kaplan Meier survival curve was created and statistical analysis was performed using Log-rank (Mantel-Cox) test by GraphPad Prism 8.0 software. A *p* value of <0.05 was considered as statistically significant.

Results

Dermal injection of CD11b⁺ myeloid cells isolated from AA-affected skin induces AA in healthy C3H/HeJ mice

We have recently developed a simple method to induce AA in healthy C3H/HeJ mice by dermal injection of 1-3 million of non-cultured or cultured adherent cells isolated from AA-affected skin ²³. Using this method, we have noticed that CD11b⁺ myeloid cells which count for 10% of injected cells might play a role in induction of AA in healthy C3H/HeJ mice. As AA skin-derived attached cells consist of several different cell types, including dermal fibroblasts and epithelial cells, here, we have asked the question of whether CD11b⁺ cells isolated from AA-affected skin is able to induce AA in C3H/HeJ mice. Prior to cell injection, we used a CD11b positive selection kit to isolate CD11b⁺ myeloid cells from skin and spleen and showed that the CD11b⁺ isolated cells were more than 80% pure via immunofluorescent staining (Fig. 1a). The result of using these cells showed that dermal injection of only 0.5 million of AA derived CD11b⁺ myeloid cells with or without culture was sufficient to induce AA in healthy C3H/HeJ mice. In brief, 7 and 6 out of 8 mice that received dermal injection of non-cultured and cultured AA skin derived CD11b⁺ cells developed AA within 12 weeks, respectively (Fig. 1b and c). At the same time period, none of the 8 mice that received AA skin derived CD11b⁻ cultured cells showed any sign of hair loss and only 1 out of 8 mice that received dermal injection of naïve CD11b⁺ myeloid cells isolated from non-AA derived spleen developed AA in the same mouse model (Fig. 1b and c). The induction of AA in the single mouse which received naïve CD11b⁺ myeloid cells is likely to be due to spontaneous AA development as low frequency and spontaneous AA development has previously been reported in C3H/HeJ mice ²⁴. This result suggests that only CD11b⁺ myeloid cells from AA-affected skin, but neither AA derived CD11b⁻ skin cells nor naïve CD11b⁺ from non-AA spleen, are able to induce AA in C3H/HeJ mice.

To address whether the CD11b⁺ cells is also present in the skin of AA mice induced by other method, we obtained mouse skin from AA mice induced by dermal injection of 10 million of activated T lymphocytes ²⁵ and stained with CD11b antibody. The result showed a remarkable increase in the number of these cells within AA lesions as compared to those of normal control (Supplemental Fig. S1a-c). This result further supports the association of CD11b⁺ myeloid cells with AA.

Macrophage colony-stimulating factor (M-CSF) is highly expressed in AA skin cells

Based on the result in Figure 1, we realized that CD11b⁺ myeloid cells isolated from AA affected skin of mice are different with CD11b⁺ myeloid cells isolated from splenocytes of healthy mice, suggesting CD11b⁺ myeloid cells in AA skin may be activated by inflammatory factors. M-CSF is a regulator for macrophage polarization and proliferation²⁶ and its expression is high in inflamed tissue²⁷. Therefore, we next examined whether M-CSF is expressed in AA affected skin and whether it is the factor to activate naïve CD11b⁺ myeloid cells to become AA-induction immune cells. As shown in Fig.2a, our result revealed a remarked increase in the number of M-CSF positive cells in AA skin as compared to that in normal skin. We then examined which cells expressed M-CSF. Previous study has demonstrated that the expression of M-CSF is strictly controlled by cell proliferative state²⁸. We therefore performed double immunofluorescent staining with M-CSF and Ki67 antibodies in AA affected skin of mice. Indeed, the most of Ki67 positive cells were expressed M-CSF (Fig. 2b). To further examine which type of cells expressed M-CSF, we isolated total cells from the skin of AA mice and cultured them for 48 hours before performing a double immunofluorescent staining with M-CSF and other antibodies. As shown in Fig. 2c, the expression of M-CSF was not limited to one type of cells. Some of CD45 or CD11b positive blood cells, P63 positive epithelial stem cells and type 1 pro-collagen positive fibroblasts expressed M-CSF. We then compared the level of M-CSF expression in normal and AA mouse skin, detected by western blot. Skin was collected from three normal and three AA mice, skin cell mixture was isolated from the skin by collagenase digestion and protein was extracted from the cell pellet. The result demonstrated a significant increase in M-CSF protein in AA skin as compared to that in normal skin (Fig. 2d-e). This finding suggests that M-CSF, a myeloid cell stimulating factor, is highly expressed in AA skin cells.

Dermal injection of M-CSF-cultured CD11b⁺ myeloid cells induces AA in C3H/HeJ mice

A high expression of M-CSF and abundant CD11b⁺ myeloid cells in AA affected skin let us hypothesize that M-CSF-stimulated CD11b⁺ myeloid cells could induce AA in C3H/HeJ mice. To test this hypothesis, naïve splenocytes from healthy C3H/HeJ mice were cultured in the presence of M-CSF and the result showed that around 95% of adherent cultured cells were CD11b-positive cells (Fig. 3a-b). To test the AA induction, one million of M-CSF-cultured CD11b⁺ cells mixed with or without three million of freshly isolated naïve splenocytes from non-AA-affected healthy C3H/HeJ mice were dermally injected into one spot of dorsal skin of normal C3H/HeJ mice. The results showed that within 10 weeks following cell injection, 4 out of 5 mice received M-CSF-cultured myeloid cells alone and 5 out of 5 mice received myeloid cells plus naïve splenocytes developed patchy hair loss (Fig. 3c-d). None of the mice with either no treatment or injection of three million of naïve splenocytes alone showed any signs of hair loss. These results demonstrated that M-CSF-cultured CD11b⁺ myeloid cells have the capacity to induce AA similar to those isolated from AA affected skin. Further, naïve immune cells reaching AA lesion might become activated and play an important role in development of chronic inflammation seen in AA affected skin.

AA skin-associated and M-CSF-cultured CD11b⁺ myeloid cells are able to activate naïve splenocytes *via* releasable factors

To explore the mechanism of how these CD11b⁺ myeloid cells can induce AA in vivo, next, we test whether these cells can directly or indirectly activate other immune cells. We used a co-culture experiment to test this hypothesis. First, we evaluated the percentage of CD11b⁺ cells in a mixture of cells isolated from AA and non-AA skin by FACS analysis. As shown in Fig. S1c, the number of CD11b⁺ cells were 5 folds higher in AA skin as compared to that of control (10% vs 2% of control). We then showed that co-culturing splenocytes with a mixture of cells isolated from AA skin markedly increased the proliferation of naïve splenocytes as compared to that of control (Fig. 4a). When myeloid cells were removed from the skin cell mixture by treatment with clodronate liposome, sub-cultured them prior to co-culture with splenocytes, the proliferation of co-cultured splenocytes was remarkably reduced as compared to the splenocytes co-cultured with AA skin adherent cells (Fig. 4a). This finding suggests that there seems to be a correlation between the number of CD11b⁺ cells in AA derived mixture of cells and the splenocyte proliferation index.

To further confirm this finding, CFSE-labeled naïve splenocytes were cultured either alone, mixed with M-CSF-treated CD11b⁺ myeloid cells, co-cultured with M-CSF-treated myeloid cells separated by a trans-well chamber or cultured in a medium containing 50% conditioned medium collected from M-CSF-cultured myeloid cells. As shown in Fig. 4b, activated splenocytes were observed under the light microscope (cells aggregated to form cluster and enlarged in size) when splenocytes were co-cultured with M-CSF-treated myeloid cells or cultured in a conditioned medium from M-CSF-treated myeloid cells. Splenocyte activation was further evaluated by FACS analysis for CFSE-labeled splenocyte proliferation (Fig. 4c). The result demonstrated that naïve splenocytes were proliferative when they were co-cultured with M-CSF-stimulated CD11b⁺ myeloid cells or cultured in a medium containing 50% conditioned medium from M-CSF-stimulated CD11b⁺ myeloid cells.

M-CSF-stimulated CD11b⁺ myeloid cells activate B lymphocyte *in vitro* via releasable factor(s) and B cell infiltration is found in AA skin of C3H/HeJ mice

To examine which subset of immune cells in splenocytes were activated by the conditioned medium of M-CSF-stimulated CD11b⁺ myeloid cells, we cultured mouse naïve splenocytes in a medium containing 50% conditioned medium from M-CSF-stimulated CD11b⁺ myeloid cells. After 4 days, splenocytes were collected and stained with fluorescent-conjugated CD3 and CD19 antibodies. We noticed that activated splenocytes were enlarged in size compared to normal or naïve splenocytes (Fig 4b). Therefore, here we have gated the enlarged cells as activated splenocytes for FACS analysis. As shown in Fig. 5a, the majority of enlarged activated splenocytes were CD19 positive B cells (90% and 70% from two gating enlarged cells cultured in 50% conditioned medium, respectively). Consistent with the increase of B cells, the IgG level in conditioned medium of splenocytes was also higher when splenocytes were cultured in 50% conditioned medium of M-CSF-stimulated myeloid cells as compared to splenocytes cultured alone (Fig. 5b-c). These data suggested that releasable factors from M-CSF-stimulated CD11b⁺ myeloid cells are able to activate B cells.

To examine whether B cells in AA-affected skin and regional lymph nodes are activated, related tissues were used to perform immunofluorescent staining with antibodies of either CD3, CD19 or IgG. The result showed a remarkable increase of CD3, CD19 and IgG positive cells in AA-affected skin as compared to that in normal skin of C3H/HeJ mice (Fig. 5d). These findings suggested that both B and T lymphocytes are increased in AA-affected skin. To examine whether B cells in AA skin of mice induced by dermal injection of skin cell mixture are activated, we detected IgG positive B cells. The results shown in Fig. 5d confirmed that IgG positive cells were present in AA skin but not in normal skin of mice. An increase in IgG level in skin cell mixture isolated from AA-affected mice was also noticed as compared to that in normal skin of mice, detected by western blotting (Fig. 5e-f). These results suggested that B cell activation may be involved in M-CSF-stimulated CD11b⁺ myeloid cell-induced AA in mice.

To further evaluate the association of M-CSF-stimulated CD11b⁺ myeloid cell-induced AA with B cell activation, we collected skin, skin regional lymph nodes and spleen from 3 mice that received either nothing (no treatment) or three million of naïve splenocytes or one million of M-CSF-cultured CD11b⁺ cells or one million of M-CSF-cultured myeloid cells plus three million of naïve splenocytes as described above (Fig. 3c-d). The mice from the untreated group and the mice that received naïve splenocytes didn't show any signs of hair loss, while three mice that received either M-CSF-cultured CD11b⁺ cells alone or CD11b⁺ cells plus naïve splenocytes were AA mice. Cells from skin, regional lymph nodes and spleen were stained with fluorescent-conjugated anti-CD3, CD19, CD4 and CD8 antibodies after being isolated from skin, lymph nodes and spleen, respectively. Positive cells were counted by FACS analysis. As shown in Supplemental Fig. S2 and Table S1, in addition to an increase in infiltration of T cells (CD3, CD4 and CD8 positive cells), the number of CD19-positive B cells was also significantly increased in the skin of AA mice that received either M-CSF-cultured CD11b⁺ cells alone or CD11b⁺ cells plus splenocytes as compared to that in the skin of healthy mice (either received nothing or naïve splenocytes). Although the number of T cells increased in AA-affected skin of mice, our results from regional lymph nodes and spleen revealed that the percentage of T cells was significantly reduced, while the percentage of B cells was significantly increased in AA mice as compared to normal mice (Supplemental Fig. S3-S4, Table S2 and S3). This result was further confirmed in AA mice induced by dermal injection of 10 million of activated T cells ²⁵, as a higher B cell population was found in AA related regional lymph nodes and spleen as compared to that in normal mice (Fig. 6a-c). These data suggest that B cell activation may play a role in AA development.

Discussion

In this study, we have demonstrated that dermal injection of AA skin-derived CD11b⁺ but not naïve CD11b⁺ myeloid cells induces AA in C3H/HeJ mice. Interestingly, M-CSF-cultured splenocyte-derived CD11b⁺ cells function the same as AA skin derived CD11b⁺ cells in induction of AA in the same mouse model. To explore the possible mechanisms, we demonstrated that both AA skin derived and M-CSF-cultured CD11b⁺ myeloid cells are able to activate B lymphocytes *via* releasable factor(s).

Myeloid cells such as monocytes, macrophages, dendritic cells and neutrophils have long been known to be involved in innate immune activation and tissue inflammation ^{29, 30}. Myeloid cells are also known to play a crucial role in the development of many autoimmune diseases ³¹. In this study, we demonstrated that isolated AA skin-associated and M-CSF-cultured CD11b⁺ myeloid cells are able to induce AA in C3H/HeJ mice. These AA-associated myeloid cells need a stimulation from M-CSF, which is highly expressed in AA skin of mice, to become AA-associated inflammatory myeloid cells. M-CSF is a hematopoietic cell growth factor that regulates myeloid cell proliferation, differentiation and survival ³². Mice with M-CSF gene deficiency are resistance to collagen-induced rheumatic arthritis ³³ and dextran sulfate sodium-induced colitis ³⁴. M-CSF has also been shown to enhance the inflammatory response of fibronectin-primed macrophages ³⁵ and mediate TNF-induced inflammatory osteolysis ³⁶. All these previous studies strongly support a link between inflammatory myeloid cells and M-CSF, and the association of M-CSF-stimulated CD11b⁺ myeloid cells with AA. Further, in this study, we found that releasable factors from M-CSF-stimulated CD11b⁺ myeloid cells are able to activate other immune cells, especially B lymphocytes *in vitro* co-culture experiment. B lymphocyte activation is also demonstrated in skin draining lymph nodes and spleen of AA mice. We speculate that B cell activation may be involved in M-CSF-stimulated CD11b⁺ myeloid cell-induced AA development in C3H/HeJ mice.

Although, there is convincing evidence that T cells are involved in AA induction, our results suggest that B cells are also likely to be directly or indirectly involved in AA induction as well. This is because regardless of the method used to induce AA in mice, the majority of the expanded immune cells in lymph organs (skin draining lymph nodes and spleen) are B cells. The number of B cells and IgG-positive cells were also detected in AA skin of mice, indicating B cells are activated in AA mice. Importantly, we found that releasable factors from AA derived CD11b⁺ myeloid cells mainly activate B cells and not T cells in a co-culture experiment. These *in vivo* and *in vitro* data strongly support a link between AA and B cell activation. However, we are still unsure of the exact role that B cells play in AA development based on the current data. Further study is needed to see whether depletion of B cells by antibody or genetical modification can affect AA development.

In conclusion, here, we have provided compelling evidence indicating that AA can be induced by AA skin-associated or M-CSF-stimulated CD11b⁺ myeloid cells. M-CSF-stimulated CD11b⁺ myeloid cells seem able to activate B cells through unknown releasable factors. These findings may also provide insights in understanding the cellular mechanisms involved in AA and other autoimmune diseases.

Abbreviations

AA, alopecia areata; M-CSF, macrophage colony-stimulating factor; CK14, cytokeratin 14; INV, involucrin; iNOS, inducible nitric oxide synthase; FACS, fluorescence-activated cell sorting; CFSE, carboxyfluorescein succinimidyl ester.

Declarations

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AUTHOR CONTRIBUTIONS

Y. L. conceived the overall project, designed and conducted most of the experiments, analysed the data, and wrote the manuscript. R.T.K. performed the animal experiments, cell culturing, and immunostaining. R.A. performed immunofluorescent staining and edited the manuscript. A. S. performed immunofluorescent staining. A. G. supervised the study and contributed to the experimental design, writing of the manuscript and supervising all the works.

CONFLICT OF INTEREST

The authors state no conflict of interest

DATA AVAILABILITY STATEMENT

No dataset were generated or analyzed during the current study. Derived data supporting the findings of this study are available from the corresponding author on request.

ARRIVE guidelines statement

We confirmed that the study is reported in accordance with arrive guidelines.

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Figures

Li et al., Figure 1a-b

Li et al., Figure 1c

Figure 1

Dermal injection of CD11b⁺ cells from AA-affected skin induces AA in C3H/HeJ mice. (a)

Immunofluorescent staining of CD11b-positive cells in purified naïve and AA-skin isolated CD11b⁺ cells. Splenocytes and collagenase digested skin cell mixture from AA skin of C3H/HeJ mice were purified by CD11b⁺ isolation kit, fixed with 10% formalin and stained with CD11b⁺ antibody (green). DAPI (blue) was used for a nuclear counterstain. Scale bars, 50 μ m. (b) Kaplan-Meier survival curve for AA induction after mice received 0.5 million of CD11b⁻ or CD11b⁺ cells for 10 weeks. Naïve CD11b⁺ myeloid cells were obtained from splenocytes of healthy C3H/HeJ mice through purification by CD11b⁺ cell isolation kit. Skin cell mixture was isolated from AA skin of mice by collagenase digestion. Cell mixture was either directly used for CD11b⁺ purification or cultured for 48 hours before isolation of CD11b⁺ and CD11b⁻

cells. Around 0.5 million indicated cells in 100 μ l medium were dermally injected into dorsal skin of healthy mice at a single spot. (c) Clinical appearances of C3H/HeJ mice receiving 0.5 million indicated cells for 10 weeks.

Li et al., Figure 2a-c

Li et al., Figure 2d-e

Figure 2

M-CSF is expressed in AA skin cells. (a) Immunofluorescent staining was used to detect M-CSF-expressed cells (red) in skin of normal and AA mice. DAPI was stained blue. Scale bars, 50 μ m; (b) Double immunofluorescent staining with M-CSF (red) and Ki67 (green) antibodies in skin of AA mice. DAPI was stained blue. Scale bars, 50 μ m; (c) Skin cell mixture were isolated from the skin of AA mice and cultured for 48 hours. Cells were fixed and performed double immunofluorescent staining by M-CSF antibody and either Ki67 or CD45 or CD11b or CK14 or P63 or type-pro-collagen antibody. DAPI was stained blue. Scale bars, 50 μ m; (d) and (e) Western blot was used to detect M-CSF protein in skin of normal and AA mice. Skin was harvested from mice and digested with collagenase. The skin cell mixture was pelleted by centrifugation, washed once with PBS and lysed in cell lysis buffer. Samples were run in SDS-PAGE and western blot was performed with anti-M-CSF antibody. β -actin was used as a loading control. (d) Images

of western blot from skin cell mixture of two normal and two AA mice. β -actin was used as a loading control; **(e)** Statistical analysis of M-CSF expression in skin of three normal and three AA mice.

Li et al., Figure 3a-c

Li et al., Figure 3d

Figure 3

Dermal injection of M-CSF-stimulated CD11b⁺ myeloid cells induces AA in C3H/HeJ mice. **(a)** and **(b)** Examination of the purity of splenocyte-derived and M-CSF-cultured adherent cells by immunofluorescent staining **(a)** and FACS analysis **(b)** with CD3, CD19 and CD11b antibodies. Splenocytes from healthy C3H/HeJ mice were cultured in a medium containing 5 ng/ml of M-CSF for 6 days. The adherent cells were stained with the indicated antibody and examined by immunofluorescent microscope and flow cytometry. DAPI (blue) was used for a nuclear counterstain. Scale bars, 50 μ m. **(c)** Kaplan-Meier survival curve for AA induction after mice received the indicated cells for 10 weeks. **(d)** Clinical appearance of hair loss in C3H/HeJ mice that received the indicated cells at week 10.

Li et al., Figure 4a

Li et al., Figure 4b-c

Figure 4

AA skin cell mixture and splenocyte-derived M-CSF-cultured CD11b⁺ myeloid cells induce naïve splenocyte activation *in vitro*. (a) CFSE-labeled naïve splenocytes were co-cultured with skin cell mixture from normal and AA mice for 4 days and the suspended cells were collected for FACS analysis. Before the co-culture, skin cell mixture was isolated from the skin of normal or AA mice by collagenase digestion and cultured for 3 days. To remove myeloid cells, 24 hours-cultured skin cell mixture was treated by adding 0.12 ml /ml of liposome and clodronate liposome (5 mg/ml). Around 20,000 cells per well were sub-cultured in a 24-well plate. After 24 hours, around 2 million of CFSE-labeled naïve splenocytes from the same strain of mice were added to the top of skin cells and continued to be cultured for 4 days. The suspended cells were collected and analysed for cell proliferation by FACS. (b) and (c) CFSE-labeled naïve splenocytes were co-cultured with M-CSF-stimulated CD11b⁺ myeloid cells or their conditioned medium for 4 days and suspended cells were examined by microscope (b) and FACS analysis (c). M-CSF-stimulated CD11b⁺ myeloid cells were generated by culturing mouse splenocytes in a medium containing 5 ng/ml of M-CSF for 6 days. To collect the conditioned medium, these cells were cultured in a medium without M-CSF for 24 hours. Around 20,000 per well of CD11b⁺ myeloid cells were sub-cultured in a 24 well plate and around 2 million of CFSE-labeled naïve splenocytes from the same strain of mice were co-cultured with M-CSF-stimulated CD11b⁺ myeloid cells together (Tog) or separated by a trans-well chamber (Sep) for 4 days. (b) Cell morphology of suspended cells under the light microscope after 4 days

co-culture. Scale bars, 50 μ m. (c) FACS analysis for CFSE-labeled cell proliferation in collected suspended cells after 4 days co-culture.

Figure 5

B lymphocytes are the dominant cells in CD11b⁺ myeloid cell-activated splenocytes and increase in AA mice. (a) FACS analysis was used to evaluate the percentages of CD3-positive T cells and CD19-positive B cells in 4 days cultured splenocytes in 50% conditioned medium from M-CSF-stimulated CD11b⁺ myeloid cells (myeloid cell CM). Gated cells (indicated activated splenocytes) based on scatter (cell size) were labeled by red and evaluated for the percentage of T and B cells. Splenocytes cultured in regular medium (inactivated splenocytes) were used as control. (b) and (c) IgG levels in conditioned medium of splenocytes cultured in regular medium or 50% conditioned medium of M-CSF-stimulated CD11b⁺ myeloid cells for 4 days, detected by western blot. CBS, Coomassie blue stain. (d) Immunofluorescent staining using CD3, CD19 or IgG antibody in skin of normal or AA mice. DAPI (blue) was used for nuclear staining. CD3, CD19 and IgG positive cells were stained red. Scale bars, 50 μ m. (e) and (f) IgG levels in skin cell mixture isolated from three normal and three AA mice. Around 80% body surface area of skin was harvested and digested by collagenase for one hour. Skin cell mixture was then pelleted by centrifugation and washed once with PBS. The cell pellet was lysed in cell lysis buffer and proteins were used for western blot with goat anti-mouse IgG conjugated HRP antibody. Only IgG in infiltrated B cells was detected by this method. β -actin was used as a loading control.

Li et al, Figure 6a-c

Figure 6

B cells and not T cells, in skin draining lymph nodes and spleen, are dominantly expanded in AA mice that received dermal injection of activated T cells. (a) Photos of lymph nodes and spleens from three normal and three AA mice; **(b)** and **(c)** CD3 and CD19 cell percentage in skin-draining lymph nodes **(b)** and spleen **(c)** based on FACS analysis. Cells were isolated in lymph nodes and spleens by mechanical damage with

the plunger of a 10 ml syringe, stained with fluorescent-conjugated CD3 and CD19 antibody and analyzed by FACS.

Supplementary Files

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