Myeloid Adherent Cells Are Involved in Hair Loss in the Alopecia Areata Mouse Model

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Alopecia areata (AA), which is defined as an autoimmune hair loss disease, has a serious impact on the quality of life for patients with AA worldwide. In this study, to our knowledge, a previously unreported method of AA induction in C3H mice has been established and validated. Using this method, we showed that dermal injection of 1–3 million of a mixture of skin cells freshly isolated from AA-affected skin induces AA in more than 80% of healthy mice. Contrary to the previous protocol, the induction of AA by this approach does not need any surgical AA skin grafting, cell manipulation, or high number of activated T cells. We also showed that dermal injection of adherent myeloid cells (mainly CD11b+) in healthy mice is as potent as a mixture of non-adherent CD3+ T cells and CD19+ B cells in the induction of AA. Interestingly, most of the mice (7 out of 8) that received non-adherent cells developed AA universalis, whereas most of the mice (5 out of 7) that received adherent cells developed patchy AA. Finally, we found a high number of stage-specific embryonic antigen-expressing cells whose expression in monocytes in an inflammatory disease causes the release of inflammatory cytokines, TNF-α and IL-1β, from these cells in AA-affected skin.


INTRODUCTION

Alopecia areata (AA) is an autoimmune and inflammatory skin disease that causes sudden hair loss. According to the area of hair loss, patients with AA are defined as alopecia patchy (patchy hair loss), alopecia totalis (whole head hair loss), or alopecia universalis (total body hair loss). It is estimated that 0.1% to 0.2% of people are affected, with a lifetime risk of 2.1% (Mirzoyev et al., 2014).

AA is an inflammatory skin disease in which T lymphocytes, including CD4+ and CD8+ T cells, usually infiltrate in the juxta-follicular area in the acute phase. In the chronic phase of AA, CD8+ T cells dominate around the hair bulbs (Ito et al., 2013). Experimental evidence showed that injections of CD8+ cells from AA mice to non-AA mice could induce hair loss at local skin (McElwee et al., 2005), whereas depletion of CD4+ or CD8+ T cells in the Dundee Experimental Bald Rat model could partially recover hair-growth (McElwee et al., 1996, 1999). Despite a functional link between activated T lymphocytes and hair loss, the potential role of adherent immune cells in AA development is not clear.

With the exception of highly controlled and regulated clinical trials for the treatment of AA, the use of humans for experimental investigation is impossible owing to obvious ethical issues. In the past, several animal models of AA have been developed, of which the most notable are the C3H/HeJ mouse model (McElwee et al., 1998) and the Dundee Experimental Bald Rat model (Michie et al., 1991). C3H/HeJ mice develop a spontaneous, complex polygenic AA-like hair loss with low incident rate (<1% in less than 6 months and around 20% by 12 months of age) (Sundberg et al., 1994). AA can be induced in C3H/HeJ mice by either skin grafting one piece of affected mouse skin to normal mice or dermal injection of 10 million non-cultured lymphocytes or cultured activated lymphocytes, which are obtained from the skin-derived lymph nodes of affected mice (McElwee et al., 2005; Silva and Sundberg, 2013; Wang et al., 2015). However, these strategies require either an invasive surgery with the risk of graft-rejection and infection (McElwee et al., 1998), a large number of cells from multiple donors sufficient only for a limited number of recipient mice (McElwee et al., 2005), or the use of expensive CD3/CD28 beads and cytokine cocktails for lymphocyte expansion (Wang et al., 2015).

In this study, to our knowledge, a previously unreported strategy has been established and validated for AA induction in C3H/HeJ mice and showed that myeloid cells isolated from AA-affected skin are as important as non-adherent lymphocytes in the induction of AA in healthy C3H mice. Evaluating the skin of these mice showed a high number of cells co-expressing stage-specific embryonic antigens (SSEAs) 1 and 3 in AA-affected skin. These SSEA-positive cells are derived from CD11b+ myeloid cells.

RESULTS

Dermal Injection of a Mixture of Cells Isolated from AA-Affected Skin Induces AA in C3H/HeJ Mice

The total number of cells isolated from AA-affected mouse skin was 74 ± 10 × 10⁶ cells (ranged from 66 × 10⁶ to 88 × 10⁶ cells, n = 4). The result of FACS analysis showed the presence of CD3+ T cells (20%), CD19+ B cells (11.5%), and...
CD11b+ myeloid cells (16.1%) in a mixture of isolated skin cells (Figure 1a). With dermal injection of either 1 or 3 million of a mixture of skin cells isolated from AA-affected mouse skin to healthy C3H/HeJ mice, around 80% of mice developed AA (Figure 1b). As shown in Table 1, none of the mice that received either nothing (negative control) or 1 million of non-AA-affected skin cell mixture developed AA within 6 months post the injection of cells.

Cultured Adherent Myeloid Cells Isolated from AA-Affected Skin Induces AA in C3H mice
To address whether adherent myeloid cells are as important as lymphocytes in the induction of AA, a mixture of total cells isolated from AA-affected skin was cultured. After 24 hours, non-adherent cells were harvested and dermally injected into 8 healthy C3H/HeJ mice, while adherent cells were gently washed 3 times with PBS and remained in the culture for another 48 hours. Before cell injection, the harvested cells were subjected to FACS analysis and the result showed the presence of 14% CD3+ T cells, 9.7% CD19+ B cells, and 6.6% CD11b+ cells in the non-adherent cell mixture, whereas 10.5% CD11b+ cells and negligible number of CD3+ T cells (1.0%) and CD19+ B cells (0.6%) were present in the adherent cell mixture (Figure 2a). The results of AA induction showed that 8 out of 8 mice that received non-adherent cells developed AA, whereas 6 out of
7 mice that received adherent cells developed AA (Figure 2b). Interestingly, most of the mice (7 out of 8) that received non-adherent cells developed AA universalis, whereas most of the mice (5 out of 7) that received adherent cells developed patchy AA, and only one mouse that received adherent cells developed AA universalis (Figure 2b and Table 1).

Stage-Specific Embryonic Antigen (SSEA)-1 and SSEA-3 Positive Cells Are Present in AA-Affected Skin

As cells expressing SSEAs, markers for pluripotent stem cells (Trusler et al., 2018), have been found in inflammatory tissues (Galassi et al., 2018; Kaminagakura et al., 2007; Kurose et al., 2017) and activate monocytes which release inflammatory cytokines, TNF-α and IL-1β (Lo et al., 1997), here, we examined whether SSEA-positive cells are present in AA-affected skin. By using immunofluorescent staining for SSEA-3, we found the presence of SSEA-3 positive cells in AA-affected skin but not in the normal skin (Figure 3a, top panels). Most of the SSEA-3 positive cells also co-expressed SSEA-1 in AA-affected mouse skin (Figure 2b).

To examine the correlation between SSEA-positive cells and adherent CD11b+ myeloid cells, we performed double immunofluorescent staining for CD11b+ and SSEA-1 and showed that some of the CD11b+ cells, but not all, are also positive for SSEA-1. This suggests that, at least, some of the cells expressing SSEAs and CD11b+ might be directly or indirectly responsible for the induction of AA in C3H/HeJ mice.

**DISCUSSION**

In this study, we have established and validated a simple model of AA induction by using dermal injection of a mixture of cells isolated from AA-affected skin without any cellular manipulation into C3H/HeJ. We also showed that adherent myeloid cells (CD11b, SSEAs positive) are as important as a mixture of non-adherent CD3+ T cells and CD19+ B cells in the induction of AA. Contrary to the previous protocol, the induction of AA in the same strain of mice does not need any surgical procedure for AA skin grafting (McElwee et al., 1998; Silva and Sundberg, 2013) or higher number of activated T cells (McElwee et al., 2005; Wang et al., 2015). Here, by using a simple procedure of isolating a mixture of total skin cells from AA-affected skin and dermally injecting them in healthy C3H mice, we showed that AA can be induced in around 80% of mice that received a mixture of cells isolated from AA-affected skin. For comparison, we have also used the previously reported protocol of AA induction in mice and found that 19 out of 42 (45.2%) mice that received dermally injected 10 million cultured activated T cells developed AA (Table 1). When using 10 times fewer cells (1 x 10^6 vs 1 x 10^7 cells) in our inducible AA model, 33 out of 39 mice (84.5%) that received isolated AA-affected skin cells developed AA (Table 1).

In a series of experiments, we have evaluated the role of adherent myeloid cells (CD11b) in the AA induction and showed that cultured adherent cells isolated from AA-affected skin also induce AA with a similar success rate as that of non-adherent cells. This finding suggests that adherent cells have the capacity to activate naive T cells responsible for hair loss either directly at the site of injection or indirectly through migration to regional lymph nodes. This shows that dermally injected adherent cells causing patchy AA supports the idea that these cells might directly activate T cells at the site of injection. The result of FACS analysis revealed that the majority of adherent cells are positive for CD11b with a small number of T and B cells. Knowing that the association of SSEA-1 and SSEA-3 cells with monocytes in inflammatory diseases causes the release of inflammatory cytokines such as TNF-α and IL-1β (Galassi et al., 2018; Kaminagakura et al., 2007; Kurose et al., 2017), we examined the presence of these cells in AA-affected skin. The result showed a high number of SSEA-1 and SSEA-3 cells in AA-affected skin (Figure 3), some of which were also positive for CD11b in the cultured adherent cells. However, it is not clear whether SSEA-positive adherent cells directly or indirectly activate T cells and monocytes in AA-affected skin or are co-expressed with CD11b without being involved in the development of AA. For this reason, more study is needed to investigate the potential role of SSEA-positive myeloid cells in the development of AA.

In summary, here, we have established a reproducible and simple method to induce AA in C3H/HeJ mice and showed that adherent myeloid cells (CD11b, SSEAs) are as important as a mixture of non-adherent CD3+ T cells and CD19+ B cells in the induction of AA. Interestingly,
alopecia universalis and patchy are induced in healthy C3H mice by dermally injected non-adherent and adherent cells isolated from AA-affected mouse skin, respectively.

**Materials and Methods**

**Animals**

Female C3H/HeJ mice aged nine weeks were purchased from The Jackson Laboratory (Bar Harbor, Maine). The University of British
Columbia Animal Care Committee approved all experimental procedures. The methods were carried out in accordance with the approved guidelines. Mice were socially housed under a 12 hour light/dark cycle.

**Cell Isolation from AA-Affected Mouse Skin**

As a source of AA-affected skin, we first induced AA by using previously published protocol (Wang et al., 2015). Subsequently, AA was induced by using our established protocol. To isolate cells from mouse skin, about 80% of the skin from euthanized mice with AA universalis was collected, cut into small pieces, and washed 3 times in PBS with 3% antibiotic-antimycotic (Invitrogen Life Technologies, Burlington, ON, Canada). Minced skin was incubated in PBS with 1 mg per ml of collagenase V (Sigma-Aldrich, St. Louis, MO) at 37°C for 90 minutes. After the neutralization of collagenase with equal volume of DMEM (Invitrogen Life Technologies) containing 10% fetal bovine serum (Invitrogen Life Technologies), the extract was filtered through a 70 μm cell strainer. Cells were pelleted by

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**Figure 3.** SSEA-1 and SSEA-3 positive myeloid cells are present in AA-affected skin of C3H/HeJ mice and cultured adherent cells isolated from AA-affected skin of mice. (a) Detection of SSEA-3 positive cells in AA-affected skin as compared with that in non-AA-affected skin. (b) Double immunofluorescent staining with SSEA-1 and SSEA-3 antibodies in AA-affected skin. (c) Double immunofluorescent staining with CD11b and SSEA-1 antibodies in cultured adherent cells isolated from AA-affected skin of mice. DAPI (blue) was used as a nuclear counterstain. AA, alopecia areata; SSEA, stage-specific embryonic antigen. Bar = 50 μm.
centrifugation (800 g, 10 minutes) and washed once with DMEM containing 10% fetal bovine serum. Skin cell mixture was either directly dermally injected into healthy C3H/HeJ mice or cultured in DMEM containing 2% fetal bovine serum.

**FACS Analysis**

For FACS analysis, the following antibodies were used: anti-mouse CD3e-PE (1:100, Invitrogen Life Technologies), anti-mouse CD19-APC (1:100, Invitrogen Life Technologies), and anti-mouse CD11b-PE (1:100, Invitrogen Life Technologies). Cells were stained with antibody overnight. For culture cells, after washing once with PBS, cells were fixed with 10% formalin for 30 minutes, washed by PBS, and blocked with blocking buffer. The following primary antibodies were incubated with a blocking buffer (5% of bovine serum albumin in PBS) for 1 hour before incubation with the primary antibody overnight. For culture cells, after washing once with PBS, cells were fixed with 10% formalin for 30 minutes, washed by PBS, and blocked with blocking buffer. The following primary antibodies were used: SSEA-1 (MC480) (1:100, Cell Signalling Technologies, Whitby, ON, Canada), SSEA-3 (1:1000, R & D Biosystem, Minneapolis, MN), and CD11b (1:500, eBioscience, San Diego, CA).

**Data availability statement**

The data that support the findings of this study are available from the corresponding author, AG, on request.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: YL and AG; Data Curation: YL; Formal analysis: YL; Funding Acquisition: AG; Investigation: YL, RTK, GL, AG; Methodology: YL, RTK, GL; Project Administration: AG; Resources: AG; Supervision: AG; Validation: YL, AG; Writing-Original Draft Preparation: YL, AG; Writing-Review and Editing: RTK, AG.

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