The Importance of Myeloid-Derived Suppressor Cells in the Regulation of Autoimmune Effector Cells by a Chronic Contact Eczema

Rachid Marhaba, Mario Vitacolonna, Dagmar Hildebrand, Michal Baniyash, Pia Freyschmidt-Paul and Margot Zöller

J Immunol 2007; 179:5071-5081; doi: 10.4049/jimmunol.179.8.5071
http://www.jimmunol.org/content/179/8/5071

References
This article cites 62 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/179/8/5071.full#ref-list-1

Subscription
Information about subscribing to J Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Importance of Myeloid-Derived Suppressor Cells in the Regulation of Autoimmune Effector Cells by a Chronic Contact Eczema

Rachid Marhaba,* Mario Vitacolonna,* Dagmar Hildebrand,* Michal Baniyash,‡ Pia Freyschmidt-Paul,§ and Margot Zöller2*†

Induction of a chronic eczema is a most efficient therapy for alopecia areata (AA). We had noted a reduction in regulatory T cells during AA induction and wondered whether regulatory T cells may become recruited or expanded during repeated skin sensitization or whether additional regulatory cells account for hair regrowth. AA could not be cured by the transfer of CD4+/CD25hi/b lymph node cells from mice repeatedly treated with a contact sensitizer. This obviously is a consequence of a dominance of freshly activated cells as compared with regulatory CD4+/CD25+ T cells. Instead, a population of Gr-1+/CD11b+ cells was significantly increased in skin and spleen of AA mice repeatedly treated with a contact sensitizer. Gr-1+/CD11b+ spleen cells mostly expressed CD31. Expression of several proinflammatory cytokines as well as of the IFN-γ receptor and the TNF receptor 1 were increased. Particularly in the skin, Gr-1+ cells expressed several chemokines and CCR8 at high levels. Gr-1+/CD11b+ cells most potently suppressed AA effector cell proliferation in vitro and promoted partial hair regrowth in vivo. When cocultured with CD4+ or CD8+ cells from AA mice, the Gr-1+/CD11b+ cells secreted high levels of NO. However, possibly due to high level Bcl-2 protein expression in AA T cells, apoptosis induction remained unaltered. Instead, ζ-chain expression was strongly down-regulated, which was accompanied by a decrease in ZAP70 and ERK1/2 phosphorylation. Thus, a chronic eczema supports the expansion and activation of myeloid suppressor cells that, via ζ-chain down-regulation, contribute to autoreactive T cell silencing in vitro and in vivo. The Journal of Immunology, 2007, 179: 5071–5081.

Immune responses directed against pathogens and self-modified Ags occur following the initial step of specific Ag recognition and the transmission of activating signals mediated by the TCR complex. After Ag elimination, the responding T cells are turned off and respond to a secondary antigenic stimulation only after a resting period. T cells have an array of shut-off mechanisms, one of which involves expression of the TCR itself. Following TCR-mediated activation, the entire TCR complex is internalized and all of its subunits are degraded in the lysosome (1–3). The cells remain unresponsive for up to 72 h or longer (3, 4). Other shut-off mechanisms are the inhibitory coreceptor CTLA4 (5), the E3 ligase CBL (6), the protein tyrosine kinase Csk, and the phosphatase SHP1 (7, 8).

Various subsets of hemopoietic cells and/or derived factors are involved in these immunoregulatory processes. Attention has recently been focused on regulatory CD4+CD25+FoxP3+ T cells, which primarily function by cell-cell contact to maintain homeostasis of the immune system (9, 10). However, these cells are not equipped with the appropriate machinery to suppress activated T cells that have expanded in response to excessive stimuli (11). Accordingly, the transfer of regulator T cells (Treg)3 can prevent autoimmune disease exacerbation but is rarely curative in the florid disease state (10, 11).

Defects in T cell activation have also been noted in chronic infections and cancer (12–16) where the sustained stimulation of the immune system leads to down-regulation of TCR ζ-chain expression and impaired T cell function (17). There is evidence for a critical involvement of IFN-γ, which most likely supports generation and/or recruitment of a myeloid-derived Gr-1+CD11b+ suppressor cell (MDSC) (17) responsible for ζ-chain down-regulation (18–20). MDSC negatively affect T cell expansion and effecter functions as well as NK cells, which express the ζ-chain (21–25). Although this type of a self-regulatory mechanism is disadvantageous in chronic diseases as well as in therapeutic settings based on repeated vaccinations (12, 13, 26), it could potentially be advantageous in controlling overshooting immune reactions as in autoimmune diseases.

We aimed to assess this hypothesis in a therapeutic setting for the autoimmune disease alopecia areata (AA). AA is a mild, non-lymph autoimmune disease (27) that affects anagen stage hair follicles (28, 29). Therapy can be based on corticosteroids (30), but induction of a chronic eczema is the most efficient therapeutic regimen in humans (31, 32). Similarly, a murine AA model,
closely mimicking the human disease (33), can be cured by repeated applications of the contact sensitizer squaric acid dibutyl ester (SADBE) (34). Although there is evidence for impaired T cell responsiveness in mice displaying hair regrowth after prolonged SADBE treatment (35), the underlying mechanism(s) are still disputed. As outlined above, persistent stimulation of the immune system with a contact sensitizer could be accompanied by the generation and recruitment of MDSC, which, in turn, could control autoimmune reactive T cells. Should our hypothesis prove to be relevant, MDSC may be a means for a tailored therapy in autoimmune diseases by inducing T cell dysfunction associated with \( \zeta \)-chain down-regulation. Impaired T cell activation in persistently SADBE-treated AA mice, indeed, is due to MDSC expansion and activation.

**Materials and Methods**

**Mice and treatment**

C3H/HetJ mice from The Jackson Laboratory, Bar Harbor, Maine, received autoclaved food pellets and acidified water ad libitum.

AA was induced by the transfer of a full-thickness skin graft from spontaneously AA-affected mice to mice with normal hair (36). Within 6 wk, roughly 90% of grafted mice develop AA. Mice with or without AA were sensitized with 2% SADBE in acetone (1.0 \( \times \) 1.0 cm area of the back) followed by weekly topical applications of 0.5 or 1% SADBE in acetone on the back and the abdominal wall to induce a moderately severe contact dermatitis lasting for 2–3 days (34). Mice were challenged 4–6 times and sacrificed 3 days after the last challenge, i.e., in AA mice the process of hair regrowth was still ongoing, complete hair regrowth requiring 8–12 wk. Animal experiments were approved by the animal health care governmental authorities of Baden-Württemberg, Germany.

**Tissue preparation**

Mice were killed by cervical dislocation. Dorsal skin samples were embedded in OCT compound (Tissue-Tek; Sakura) and snap frozen in liquid nitrogen. Skin-infiltrating leukocytes (SKIL) were isolated as described (37). Single cell suspensions from skin draining lymph nodes and spleens were prepared by pressure through fine gauze. CD4\(^+\) CD25\(^-\), CD8\(^-\), and CD4\(^+\) lymph node cells (LNC), Gr1\(^-\), CD11b\(^-\), and Gr-1 CD11b\(^+\) spleen cells (SC) were enriched by magnetic bead sorting (Miltenyi Biotec). To select for CD4\(^+\) CD25\(^{a/b}\) cells, relatively low amounts of CD25-PE magnetic beads were used (purity, ~90%). For the selection of Gr-1 CD11b\(^+\) cells, strongly adherent monocytes (CD11b\(^{a/b}\)) were depleted by seeding SC on petri dishes suited for macrophage (M\(\delta\)) recovery (Corn- ing). After 1 h at 37°C, nonadherent cells were vigorously washed off. Adherent cells (~90% CD11b\(^{a/b}\)) were recovered with a rubber policeman. Nonadherent cells were subjected to magnetic bead sorting of Gr-1 CD11b\(^+\) cells (purity: ~90%). Cell viability was ~70% in SKIL, 80%–90% in CD11b\(^{a/b}\) M\(\delta\), and 95%–98% in the remaining populations.

**Antibodies**

The following Abs were used: anti-mouse CD3e, CD4, CD8, CD11b (European Animal Cell Culture Collection), CD25, panCD44 (American Type Culture Collection); CD11c, CD16/32, CD31, CD43, CD120a, CD120b, CD152, GITR, FoxP3, \( \zeta \), \( \alpha \beta \)-TCR, \( \gamma \delta \)-TCR, Gr-1, IL-1e, IL-6, IL-10, IL-12; IFN-\( \gamma \), TNF-\( \alpha \), TGF-\( \beta \), CCL1, CCL2, CCL3, CCL5, CCL9, CCL20, osteopontin (OPN), GM-CSF, CCR8, ERK1.2, pERK1.2, ZAP70, JAK3, Src, phosphotyrosine, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-x\(_L\), and secondary reagents (HRP-, FITC-, PE-, or allophycocyanin-labeled anti-rat IgM, anti-rat, anti-rabbit, anti-hamster, anti-mouse IgG or streptavidin (Dianova, BD Biosciences, Biотrend).

**Flow cytometry**

Flow cytometry followed routine procedures. Negative controls were incubated with a nonbinding primary Ab and the appropriate secondary reagent. For intracellular staining, cells were fixed and permeabilized. Analysis was performed with a FACSCalibur flow cytometer and the CellQuest program (BD Biosciences). Contaminating keratinocytes in the SKIL preparation and cell debris were excluded by gating. Means \( \pm \) SD of at least three experiments are reported. Significance was evaluated using the two-tailed Student’s \( t \) test.

**Cell transfer**

Unseparated LNC or SC, CD4\(^+\) CD25\(^-\), CD4\(^+\) CD25\(^+\), or CD8\(^-\) LNC, or Gr-1 CD11b CD11c (1–2 \( \times \) 10\(^5\)) were s.c. injected and distributed over the dorsal skin of AA mice. Mice were observed for hair regrowth for 8–12 wk.

**Proliferation assay**

Cells (2 \( \times \) 10\(^3\)) were stimulated with anti-CD3e (10 \( \mu \)g/ml), Con A (7.5 \( \mu \)g/ml), SADBE (0.1% solution), or PMA (10 \( \mu \)M) plus ionomycin (10 \( \mu \)M); T cells were cocultured with Gr-1 CD11b\(^+\) cells at a ratio of 1:1 if not indicated differently. Proliferation was determined after 48 h by \([\text{H}]\)thymidine uptake. Mean \( \pm \) SD of triplicates are shown. Significance was calculated by the two-tailed Student’s \( t \) test.

**ELISA**

The relative amount of IFN-\( \gamma \) and TNF-\( \alpha \) in culture supernatant was determined in a sandwich ELISA according to standard procedures.

**NO production**

NO production was measured with the Griess reaction in cell-free supernatants.

**Cell lysis and immunoblotting**

Cells were collected from cocultures (48 h at 37°C), washed with ice-cold PBS, and lysed (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM Na\(_2\)VO\(_4\), 10 mM NaF, 1% Triton-X-100, 1 mM PMSF, and a protease inhibitor mix). After 30 min at 4°C the lysates were centrifuged (15000 \( \times \) g for 10 min at 4°C) and supernatants were collected. Protein content was normalized and 30 \( \mu \)l of lysates were resolved by electrophoresis on 10 or 12% for CD3\(\gamma\) SDS-polyacrylamide gels under reducing conditions. After protein transfer to nitrocellulose membranes (30 V for 16 h at 4°C), membranes were blocked (PBS, 5% BSA, and 0.1% Tween 20 for 1 h at room temperature). Immunoblotting with the indicated Abs was followed by the appropriate secondary HRP-conjugated Ab (1 h at room temperature). Blots were developed with the ECL detection system.

**Results**

**\( T_{reg} \) in contact sensitizer-treated AA mice**

An efficient therapy of AA consists of the induction of a chronic eczema (31, 32). We speculated that the therapeutic effect might be supported by regulatory mechanisms of the immune system due to the persistent stimulation. AA induction by the transfer of CD4\(^+\) cells from AA mice could be prevented by the concomitant transfer of \( T_{reg} \) (31). Thus, the therapeutic efficacy could be due to an eczema-induced expansion of \( T_{reg} \).

No major differences in CD4, CD25, CD152, and FoxP3 expression were observed between AA and AA/delayed-type hypersensitivity (DTH) LNC, although both showed a moderate increase in CD25\(^+\) cells. In SKIL, the percentage of CD152\(^+\), and FoxP3\(^+\) cells was also increased. However, in neither LNC nor SKIL were CD25\(^+\), CD152\(^+\), or FoxP3\(^+\) cells selectively increased in AA/DTH mice as compared with AA mice (Fig. 1A). Furthermore, triple fluorescence staining revealed that only the percentage of freshly activated CD4\(^+\) CD25\(^+\) CD154\(^+\) T cells was significantly increased in AA and AA/DTH LNC, although both showed a moderate increase in CD25\(^+\) cells. In SKIL, the percentage of CD152\(^+\), and FoxP3\(^+\) cells was also increased. However, in neither LNC nor SKIL were CD25\(^+\), CD152\(^+\), or FoxP3\(^+\) cells selectively increased in AA/DTH mice as compared with AA mice (Fig. 1A).

Thus, in AA and AA/DTH LNC freshly activated T cells dominate. In SKIL higher numbers of both activated and regulatory T cells were recovered in all three groups of diseased mice with no particular \( T_{reg} \) increase in AA/DTH mice.

**Inefficacy of \( T_{reg} \) from SADBE-treated mice to interfere with AA progression**

Although there was no evidence for a selective increase in \( T_{reg} \) by chronic SADBE treatment of AA mice, \( T_{reg} \) from diseased mice could display increased efficacy. However, \( T_{reg} \) from...
healthy mice were the most efficient in suppressing the proliferation of CD4^+CD25^- or CD8^+ cells (data not shown) from healthy and diseased mice. T_{reg} from AA and AA/DTH mice did not suppress proliferation of CD4^+ cells from healthy or diseased mice (Fig. 2A).

To exclude therapeutic efficacy of T_{reg} from AA/DTH mice in vivo, AA mice received s.c. injections of unseparated SC or unseparated, CD8^+, CD4^+CD25^-, or CD4^+CD25^{high} LNC from AA/DTH mice at eight different sites on the back. After 12 wk, only two of 10 AA mice showed hair regrowth after the transfer of T_{reg} from AA/DTH mice. Similar rates of hair regrowth (2–3 of 10 mice) were seen after the transfer of CD4^+CD25^-, CD8^+, or unseparated LNC from AA/DTH mice. In these four groups, sparse hair regrowth at the injection site was seen in one mouse. Instead, hair regrowth was efficiently induced by the transfer of unseparated SC from AA/DTH mice with dense hair regrowth in five and sparse hair regrowth in two of nine AA mice (Fig. 2B). In AA mice receiving PBS, sparse hair regrowth was observed in roughly 10% during an observation period of 6 mo (data not shown).
Thus, the curative effect of a chronic eczema in AA is unlikely due to T<sub>reg</sub> activation. In contrast, SC of AA/DTH mice, which were curative in 56% of mice, might contain cells that hamper AA persistence.

Characterization of MDSC in AA and AA/DTH mice

Sustained activation of the immune system may be accompanied by the induction of a population of Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSC (12–14, 26). AA is characterized by a strong increase in highly activated SkIL and skin draining LNC (38). A therapeutic effect of a contact sensitizer requires repeated application for a prolonged period (35). These features are compatible with driving the immune system into exhaustion.

In fact, Gr-1<sup>+</sup> and Gr-1<sup>+</sup>CD11b<sup>+</sup> cells were strongly increased in SC and SkIL but not in LNC of DTH and AA/DTH mice. In DTH mice, part of the Gr-1<sup>+</sup> cells expressed CD11b at a high level (Fig. 3, A and B). Thus, SADBE treatment promotes the expansion of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells in AA mice. The majority of the Gr-1<sup>+</sup> SC are CD43<sup>+</sup> (data not shown) and, particularly in diseased mice, CD44<sup>+</sup> and CD31<sup>+</sup> (Fig. 3, C and D).

Sever proinflammatory and inflammatory cytokines were expressed at increased frequency in SC and SkIL of diseased mice, with the highest level of expression frequently seen in DTH and AA/DTH mice. There was no evidence for strong up-regulation of the immunosuppressive cytokines IL10 or TGFβ (data not shown). However, in the spleens of healthy and diseased mice a significantly higher percentage of Gr-1<sup>+</sup> than Gr-1<sup>+</sup> cells expressed IL1α, IL6, and IL12. In SkIL, a relative increase of only IL12 was seen in all four groups of mice, and a relative increase in IL1α- and IL6-expressing Gr-1<sup>+</sup> SkIL was only seen in diseased mice. In SC and SkIL, up-regulation of TNF expression in Gr-1<sup>+</sup> cells was only seen in diseased mice. On the contrary, IFN-γ<sup>+</sup> cells were mostly recovered in the Gr-1<sup>+</sup> population (Fig. 3, E and G). Because MDSCs can be stimulated by IFN-γ (18–20), we analyzed CD119 (IFN-γ receptor) expression in the different subpopulations, which was up-regulated in the Gr-1<sup>+</sup> SC and SkIL of diseased mice. Expression of TNF receptor I (TNFR1; CD120a) but not TNF receptor II (TNFRII; CD120b) (data not shown) also was increased in the Gr-1<sup>+</sup> SC and SkIL of diseased mice (Fig. 3, F and G).

Because MDSC were enriched in spleen and SkIL, chemokine expression of this potentially immunoregulatory cell population was evaluated. In the spleens of AA/DTH but not AA mice a slightly but significantly increased percentage of Gr-1<sup>+</sup> expressed...
CCL2, CCL5, and GM-CSF (data not shown). The percentage of Gr-1<sup>+</sup> SkIL of AA/DTH that expressed CCL1, CCL2, CCL3, CCL5, CCL9, CCL20, and osteopontin (OPN) was strongly increased. The Gr-1<sup>+</sup> SkIL of AA/DTH mice also expressed GM-CSF and CCR8 at increased frequency (Fig. 3H).

Increased numbers of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells in the dermis and spleens of AA/DTH mice showing significant differences as compared with the Gr-1<sup>+</sup>CD11b<sup>+</sup> SkIL and SC of control mice, such as high rates of CD31, TNFRI and chemokine expression, raised the question of whether these cells are suppressive for AA effector cells.
**Gr-1<sup>+</sup>CD11b<sup>+</sup> SC suppress T cell proliferation**

Gr-1<sup>+</sup>CD11b<sup>+</sup> leukocytes from healthy, AA, DTH, and AA/DTH mice suppressed CD4<sup>+</sup> and CD8<sup>+</sup> LNC proliferation from corresponding mice. The weakest suppression was seen in cocultures from healthy mice and strongest in cocultures from AA/DTH mice. Although Gr-1<sup>+</sup>CD11b<sup>+</sup> leukocytes suppressed T cell proliferation in response to a polyclonal TCR-mediated stimulus (anti-CD3ε) and a nominal Ag (SADBE) (Fig. 4, A and B), they did not suppress the response to PMA plus ionomycin that bypasses TCR signaling (data not shown). To assess whether the suppressive activity of Gr-1<sup>+</sup>CD11b<sup>+</sup> leukocytes from healthy and diseased mice or the susceptibility of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from healthy vs diseased mice differs, MDSC from all four groups of mice were cocultured with CD4<sup>+</sup> and CD8<sup>+</sup> LNC from AA mice. MDSC from AA/DTH mice were more suppressive than MDSC from healthy mice (Fig. 4C). However, when CD4<sup>+</sup> LNC from healthy and diseased mice were cocultured with MDSC from AA/DTH mice, CD4<sup>+</sup> LNC from diseased mice were also more susceptible to the suppressive effect of MDSC than T cells from control mice (Fig. 4D). To control the in vivo efficacy of MDSC from AA/DTH mice, AA mice received six s.c. injections of 2 x 10<sup>7</sup> Gr-1<sup>+</sup>CD11b<sup>+</sup> cells or PBS distributed over the back. Patches of dense hair regrowth were seen in five of six mice receiving Gr-1<sup>+</sup>CD11b<sup>+</sup> cells but not in mice receiving PBS. An example is shown.
particular of preactivated T cells in vitro and efficiently interfere with AA effector cell activity in vivo. Thus, we analyzed the underlying mechanism.

**Gr-1⁺CD11b⁺ SC are not cytotoxic**

Activated MDSC produce increased amounts of NO, which depends on IL1β and TNF-α production and could suppress proliferation and/or induce apoptosis (20, 21, 39, 40). Because TNFR1 and TNF-α expression was up-regulated in the Gr-1⁺ SC and SkIL of diseased mice, we evaluated NO production and T cell apoptosis induction by these MDSC.

TNF-α secretion is increased in the MDSC of DTH and AA/DTH as compared with control and AA mice (Fig. 5A). When MDSC from control or diseased mice were cocultured with CD4⁺ or CD8⁺ cells on anti-CD3-coated plates, NO secretion was strongly augmented (coculture with CD4⁺ cells, 3.17- to 4.29-fold; coculture with CD8⁺ cells, 3.53- to 8.63-fold). Although IFN-γ supports the activation of MDSC (18) and is expressed on a high percentage of AA/DTH lymphocytes (Fig. 3E), the recovery of IFN-γ in cocultures of CD4⁺ and CD8⁺ cells with MDSC on anti-CD3-coated plates is strongly reduced (Fig. 5B). This is, at least, not exclusively due to IFN-γ consumption by MDSC, because the percentage of CD8⁺ (data not shown) and CD4⁺ cells expressing IFN-γ is also reduced in cocultures with MDSC (Fig. 5C). Nonetheless, MDSC from healthy or diseased mice did not induce apoptosis. The percentage of apoptotic CD4⁺ or CD8⁺ cells measured by triple staining with anti-CD4-allophycocyanin/anti-CD8-allophycocyanin, annexin-FITC, and propidium iodide, was in the same range after 72 h of culture with or without MDSC (data not shown). However, PARP degradation, particularly of CD8⁺ cells, was increased after coculture with MDSC. Akt phosphorylation and Bcl-xL expression was unaltered (data not shown). Instead, Bcl-2 expression was high in CD8⁺ cells of AA and AA/DTH mice and was further increased by coculture with MDSC (Fig. 5D).

According to these findings direct cytotoxicity of these MDSC appears unlikely. We assume that a possible effect of the MDSC on apoptosis induction may become counterbalanced by the up-regulation of Bcl-2.

**The impact of MDSC on ζ-chain expression**

MDSC also can modulate ζ-chain expression, thus prohibiting T cell activation (13, 15, 26). ζ-Chain expression was not reduced in freshly harvested CD8⁺ SC and only slightly in freshly harvested CD4⁺ SC (data not shown) but was decreased in the CD4⁺ and CD8⁺ SkIL of diseased mice. Decreased ζ-chain expression was mostly seen in activated CD25⁺ or CD152⁺ T cells, but expression was unaltered in CD95L⁺ cells (data not shown). ζ-Chain expression was reduced in αβ-TCR⁺ CD4⁺ SkIL and most pronounced in αβ-TCR- and γδ-TCR-expressing CD8⁺ SkIL of AA/DTH mice (Fig. 6A). These data were derived from SC and SkIL, collected 3 days after the last SADBE challenge. Because ζ-chain down-regulation was mostly seen in the SkIL of AA/DTH mice, which contain activated T cells and an increased number of MDSC, but not particularly enriched in activated T cells, or
in skin-draining LNC, highly enriched in activated T cells but not in MDSC (data not shown), it became likely that MDSC account for ζ-chain down-regulation. To support this interpretation, CD4⁺ and CD8⁺ LNC from healthy and diseased mice were cultured for 24 to 72 h on anti-CD3-coated plates in the presence of spleen-derived MDSC. CD4⁺ and CD8⁺ LNC, particularly from AA/DTH mice, were susceptible to ζ-chain down-regulation, which was strongest after 48 h (Fig. 6, B and C), became visible after 24 h, and was still seen after 72 h (data not shown). Western blotting confirmed ζ-chain down-regulation, most pronounced for CD8⁺ LNC, by coculture with MDSC. ζ-Chain down-regulation was accompanied by a reduction in ZAP70 and ERK1/2 phosphorylation. In addition, tyrosine phosphorylation in general (data not shown), including that of lck, which is positioned upstream of ZAP70, was severely impaired (Fig. 7). Thus, early and late signaling events during T cell activation became affected by expansion and activation of MDSC.

Taken together, repeated stimulation with a contact sensitizer induces expansion and activation of MDSC that affect activated T cells. MDSC in AA/DTH mice act similarly as MDSC in chronic infection via down-regulation of ζ-chain expression (18). Although the mechanism of ζ-chain down-regulation remains to be explored, the model of a chronic eczema therapeutically superimposed on AA supports our hypothesis that the expansion and activation of MDSC might provide a therapeutic option in autoimmune disease.

**FIGURE 6.** The impact of Gr-1⁺ CD11b⁺ cells from healthy and diseased mice on ζ-chain expression. A, ζ-Chain expression has been evaluated in CD4⁺, CD8⁺, CD25⁺, CD152⁺, and CD4⁺/TCR αβ⁺ and CD8⁺/TCR αβ⁺ or TCR γδ⁺ SkIL of healthy and diseased mice by double or triple staining with the indicated Abs. B, ζ-Chain expression was evaluated after 48 h coculture of Gr-1⁺ CD11b⁺ cells with CD4⁺ and CD8⁺ LNC of healthy and diseased mice on anti-CD3-coated plates. Cells were double-stained with anti-CD4/anti-CD8 and anti-ζ. A and B, Mean values ± SD of five experiments are shown. Significant differences in the percentages of ζ-chain expressing cells between control and diseased mice (A) and in dependence on the presence of Gr-1⁺ CD11b⁺ cells during in vitro culture (B) are indicated by an asterisk (*). C, Representative example of ζ-chain expression in CD4⁺ and CD8⁺ LNC after 48 h of culture with Gr-1⁺ CD11b⁺ SC. CD4⁺ and CD8⁺ cells have been gated. ζ-Staining of the gated CD4⁺ and CD8⁺ cells is shown.

**FIGURE 7.** The impact of Gr-1⁺ CD11b⁺ cells from healthy and diseased mice on T cell activation. LNC of healthy and diseased mice were cocultured with Gr-1⁺ CD11b⁺ cells from healthy and diseased mice for 48 h on anti-CD3-coated plates. Cells were lysed and, after SDS-PAGE, proteins were transferred and membranes were blotted with anti-ζ/anti-ζ, anti-ZAP70/anti-pZAP70, anti-ERK1/2/anti-pERK1/2 and anti-lck/anti-plck. The ratio of ζ to ζ, pZAP70 to ZAP70, pERK1/2 to ERK1/2, and ptc to lck, as revealed by densitometry, is shown. C, Control.
Discussion
The therapy of autoimmune diseases mostly relying on corticosteroids is burdened by severe side effects (41). Thus, new therapeutic options are required. A defect in Treg being casually involved in the pathogenesis of autoimmune diseases (42) created hope that a Treg transfer may be of therapeutic benefit (43). This option was evaluated in AA, a mild autoimmune disease, which is accompanied by a striking increase in peribulbar leukocytes and skin draining lymph node cells, but no burdening alterations of the immune system (29). Furthermore, a chronic eczema can be curative (34). However, the transfer of CD4+/CD25^high T cells from AA/DTH mice was ineffective after disease exacerbation, although Treg prevent disease induction (37). Yet, there was evidence that the spleens of allergen-treated AA mice contain a cell population that interferes with AA progression. Indeed, Gr-1^-CD11b^- MDSC were depleted in the spleens and SkIL of AA mice repeatedly treated with a contact sensitizer. We speculate that a chronic eczema may provide a means for MDSC activation (44) and that MDSC may be more suited than Treg for the treatment of a progressive autoimmune disease. To substantiate this hypothesis, we characterized the appearance and functional activity of MDSC in AA/DTH mice.

**MDSC from mice with a contact eczema effectively inhibit AA effector cells**

In the peripheral blood of patients with progressive AA, the majority of CD4^-CD25^- T cells are freshly activated T cells rather than Treg (45), and this finding was confirmed for the LNC and SkIL of AA mice. This feature does not change upon repeated contact sensitizer treatment of AA mice. Accordingly, the s.c. transfer of CD4^-CD25^high LNC of SADBE-treated AA mice, which showed hair regrowth, was not curative for AA mice. Repeating the experiment with CD4^-CD25^high and cells that were depleted of CD69^high T cells to reduce the “contamination” with freshly activated T cells did not induce hair regrowth at a significantly higher rate than in control mice (data not shown). Thus, the transfer of CD4^-CD25^high T cells is not beneficial in progressive AA. Although our protocol does not allow differentiation between Treg and freshly activated T cells, the transfer of FoxP3-transduced LNC stably expressing FoxP3 at a high level also did not induce hair regrowth (P.F.-P., unpublished observations), which argues against Treg efficiently interfering with progressive AA. In contrast, the transfer of unseparated SC of AADTH mice exerted a curative effect in a reasonable percentage of mice, indicating that the spleen may contain suppressor cells that can cope with a progressive autoimmune disease. A possible candidate is the Gr-1^-CD11b^- MDSC, which is enriched in the spleen. The transfer of Gr-1^-CD11b^- MDSC from AA/DTH mice indeed provoked hair regrowth in five of six AA mice.

**Characterization of MDSC in AA mice with a chronic eczema:**

Gr-1^-CD11b^- cells were enriched in the dermis and spleens of SADBE-treated mice. However, the spleens of SADBE-treated mice also contained an increased number of activated CD11b^high Mφ. In line with other reports (46), these activated Mφ were not suppressive and could even weaken the efficacy of Gr-1^-CD11b^- suppressor cells. Therefore, CD11b^high Mφ were depleted by plastic adherence before progressing with magnetic bead separation of Gr-1^-CD11b^- cells.

As described (12, 47), spleen-derived MDSC mostly expressed CD31 (PECAM-1). CD31 plays an important role in the transendothelial migration of leukocytes (48). Whether CD31 expression in splenic MDSC contributes to their migration toward the skin remains to be explored. A considerable percentage of these MDSC also expressed TGFβ, which can be involved in NO production (40, 49) and be induced by IFN-γ (50), which supports MDSC activation (18). In fact, IFN-γ expression was increased in the Gr-1^- SkIL of AA/DTH mice, whereas IFN-γ-R (CD119) expression was augmented in spleen-derived MDSC of AA/DTH mice. In SkIL, CD119 expression was high in healthy and diseased mice. Also, a significantly higher percentage of Gr-1^- than Gr-1^+ cells expressed IL-12, but IL-12 expression did not change by repeated SADBE treatment. Thus, IL-12 may not contribute to myeloid suppression. Finally, TNF-α expression and secretion as well as TNFR1 (CD120a) expression were up-regulated in the splenic and dermal MDSC of AA/DTH mice.

The chemokine expression profile of MDSC became of interest because MDSC, which are mostly recovered from the spleen, were also enriched in the dermis of SADBE-treated AA mice, pointing toward a special recruitment by chemokine receptor expression in splenic MDSC to guide them toward their target and/or by high-level chemokine expression in the dermis and/or SkIL. In fact, the SkIL of AA/DTH mice showed high level CCR8 expression, which supports effector cell recruitment in allergic skin diseases (51). Also, chemokines supporting monocyte and mixed leukocyte recruitment were expressed in a higher percentage of Gr-1^- cells from AA/DTH than control or AA mice. The high level of chemokine expression in MDSC of AA/DTH mice and the extraordinary high level of chemokine and chemokine receptor expression in the dermis of AA/DTH mice (52) could well assist MDSC recruitment.

The expanded population of MDSC in the spleens and dermis of contact allergen-treated AA mice resembles persistent infection-induced MDSC rather than tumor-induced MDSC. The subtle differences between MDSC in the spleen and dermis, like the higher level of CD31 and CD119 expression in Gr-1^-CD11b^- SC, point toward the more mature MDSC residing in the dermis. That ζ-chain expression is significantly down-regulated in freshly harvested SkIL but hardly in freshly harvested SC supports our hypothesis.

**Activity of MDSC in contact allergen-treated AA mice:**

MDSC mainly reside in bone marrow, spleens, and peripheral blood of healthy individuals and expand upon chronic stimulation of the immune system (26, 53), displaying suppressive activity mostly toward repeatedly stimulated immune effector cells (13, 26, 53). Accordingly, Gr-1^-CD11b^-+ cells of repeatedly SADBE-treated AA mice more efficiently suppressed the proliferation of stimulated rather than nonprimed T cells. This accounted for CD4^- and CD8^- as well as TCRαβ and TCRγδ T cells.

Tumor-induced MDSC can function via increased NO or reactive oxygen species production and subsequent apoptosis induction (12, 15, 54, 55). Although TNF secretion and NO production were high in the MDSC of AA/DTH mice and PARP degradation was pronounced in CD8^- T cells when cocultured with the MDSC of AA/DTH mice, apoptosis induction by TNF-α (56) was not increased. We also did not observe CD95L up-regulation (data not shown). A counter-regulation of TNF-induced apoptosis by the anti-apoptotic PI3K/Akt pathway appears unlikely, because neither Akt phosphorylation nor Bcl-xL expression were altered in T cells cocultured with the MDSC of AA/DTH mice, whereas IFN-γ expression was increased in T cells cocultured with the MDSC of AA/DTH mice (data not shown). However, Bcl-2 expression was strongly increased in T cells from AA/DTH mice and did not become mitigated by coculture with MDSC. Increased Bcl-2 expression can be due to increased NFκB activity, transducing anti-apoptotic signals (57). Among other ways, NFκB can be stimulated via the TNFR and its expression as well as TNF production are increased in AA, DTH, and AA/DTH leukocytes. By engaging TNFRI, TNF activates the transcription
factors NFκB and AP1, leading to the induction of proinflammatory and antiapoptotic genes (58). Whether activation of the NFκB pathway through the TNFRI or another receptor accounts for the observed Bcl-2 up-regulation and apoptosis resistance remains to be explored. Thus, unaltered apoptosis resistance of T cells cocultured with MDSC of AA/DTH mice may be the net result of pronounced apoptosis resistance of these T cells and apoptosis induction by MDSC.

Tumor-induced MDSC also can act via expansion of Treg (50, 59). We noted a slight increase in FoxP3 and CD152 expression in the SkIL of AA/DTH and also AA mice. Thus, although not excluded, it is unlikely that these MDSC acted via Treg expansion.

Alternatively, MDSC can induce long-lasting down-regulation and intracellular degradation of ω-chain expression resulting in a prolonged refractory period of T cells (13, 18, 21, 60) that is obvious the mechanism of action of MDSC activated by repeated SADBE treatment of AA mice. ω-Chain expression was most strongly reduced in freshly harvested CD8+ SkIL of AA/DTH mice, CD8+ T cells being the hair follicle-destructing effectors in AA (27). The overall ω-chain down-regulation was very weak in the spleen but distinct in freshly activated T cells expressing CD25 or CD152 (data not shown), which is in line with MDSC preferentially targeting activated T cells (12, 13). Also, after in vitro coculture of T cells with MDSC, IFN-γ recovery was significantly reduced. Because IFN-γ is essential for AA induction (61), the impact of MDSC on IFN-γ secretion could contribute to the therapeutic efficacy of SADBE treatment on AA persistence. IFN-γ, in contrast, supports MDSC activation (18–20). These opposing features, the suppression of IFN-γ secretion by MDSC that profit from IFN-γ, may explain why we did not observe complete ω-chain down-regulation in AA/DTH mice as described for chronic infections (13, 16, 18). Nonetheless, the strongly reduced ω-chain expression in T cells of AA/DTH mice is apparently the most relevant pathway of myeloid suppression in a persisting eczema. In line with these results was the reduced ZAP70 and ERK1/2 phosphorylation, downstream signaling components of the pathway through the TNFRI or another receptor accounts for the 

References


