Transfer of CD8$^+$ Cells Induces Localized Hair Loss Whereas CD4$^+$/CD25$^-$ Cells Promote Systemic Alopecia Areata and CD4$^+$/CD25$^+$ Cells Blockade Disease Onset in the C3H/HeJ Mouse Model

Kevin J. McElwee,*[1] Pia Freyschmidt-Paul,† Rolf Hoffmann,‡ Sabine Kissling,† Susanne Hummel,§ Mario Vitacolonna,§ and Margot Zöller§[1]

*Division of Dermatology, University of British Columbia, Vancouver, BC, Canada; †Department of Dermatology, Philipp University, Marburg, Germany; ‡Department of Dermatology, Albert-Ludwigs-University, Freiburg, Germany; §Department of Tumor Progression and Tumor Defense, German Cancer Research Center, Heidelberg, Germany

Alopecia areata (AA) is a suspected hair follicle specific autoimmune disease. The potential for cell transfer of AA using the C3H/HeJ mouse model was examined. Cells isolated from lymph nodes and spleens of AA-affected mice using magnetic bead conjugated monoclonal antibodies were subcutaneously injected into normal C3H/HeJ recipients. Within 5 wk, all CD8$^+$ cell-injected mice exhibited localized hair loss exclusively at the site of injection that persisted until necropsy. In contrast, some CD4$^+$ and CD4$^+$/CD25$^-$ cell-injected mice developed extensive, systemic AA, and a combination of CD8$^+$ and CD4$^+$/CD25$^-$ cells injected yielded the highest frequency of systemic AA induction. CD4$^+$/CD25$^+$ cells were less able to transfer the disease phenotype, partially blockaded systemic AA induction by CD4$^+$/CD25$^-$ cells, and prevented CD8$^+$ cell-induced, injection site-localized hair loss. CD11c$^+$ and CD19$^+$ cells failed to promote significant phenotype changes. Increases in co-stimulatory ligands CD40 and CD80, plus increased leukocyte apoptosis resistance with reduced CD95, CD95L, and CD120b expression, were associated with successful alopecia induction. The results suggest that CD8$^+$ cells may be the primary instigators of the hair loss phenotype. However, systemic disease expression fate is, apparently determined by CD4$^+$/CD25$^-$ cells, while CD4$^+$/CD25$^+$ lymphocytes may play a predominantly regulatory role.

Key words: autoimmune diseases/disease models/inflammation
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Alopecia areata (AA) is a suspected autoimmune disease where the clinical hair loss phenotype is believed to be induced by an inflammatory infiltrate, predominantly comprising CD4$^+$ and CD8$^+$ lymphocytes, focused on anagen stage hair follicles. Although much circumstantial evidence is consistent with this claim, parameters to define AA as a true autoimmune disease have yet to be fulfilled (McElwee *et al*, 1999b). A key requirement of Koch's postulates, as adapted to autoimmune disease, states that autoantibodies and/or autoreactive T cells are the presumed instigators of autoimmunity. The transfer of these factors to susceptible animals should lead to induction of the autoimmune disease (Rose and Bona, 1993). Intriguingly, two case reports suggest that AA might be transferred between humans by bone marrow transplantation (Barahmani *et al*, 2003; Sanli *et al*, 2004).

In other autoimmune disease models, transfer of the disease from affected to unaffected individuals using cell subsets derived from the immune system has helped define pathogenic mechanisms involved. Most commonly, CD4$^+$ (T helper, Th) lymphocytes have been identified as the primary pathogenic cell subset retaining the ability to transfer many autoimmune diseases, but other subsets including CD8$^+$ cells have also been identified as important in disease pathogenesis (Miller *et al*, 1988; Mikecz *et al*, 1990; Taylor *et al*, 1995; Petrow *et al*, 1996; Wong *et al*, 1996; Ablamunits *et al*, 1999; Mora *et al*, 1999; Huseby *et al*, 2001). In addition, regulatory cell subsets from the CD4$^+$ population, particularly CD4$^+$/CD25$^+$ cells, have been characterized with key roles in determining an individual's autoimmune disease susceptibility (Suri-Payer *et al*, 1998; Levings *et al*, 2001; Shevach, 2002). Cell subset transfer models of autoimmune disease reveal a complex interplay between pathogenic and regulatory cells of the immune system that defines the disease (Sakaguchi *et al*, 1995; Suri-Payer *et al*, 1998; McHugh and Shevach, 2002).

In rodent models for AA, depletion of CD4$^+$ or CD8$^+$ cells using monoclonal antibodies (mAb) has demonstrated that these cell subsets are fundamentally required for the perpetuation of the disease state and removal of one or the

Abbreviations: AA, alopecia areata; APC, antigen presenting cell; IFN, interferon; IL, interleukin; mAb, monoclonal antibodies; MHC, major histocompatibility complex; PE, phycoerythrin; PI, propidium iodide; SkIL, skin infiltrating leukocytes; Th, T helper cell; TNF, tumor necrosis factor

[1]These authors contributed equally to this work.

other subset enables hair regrowth (McElwee *et al*, 1996, 1999a; Carroll *et al*, 2002). Human AA-affected, skin-derived cells have been shown to reinduce inflammatory hair loss in xenografts of previously AA-affected skin grafted to *scid/scid* mice (Gilhar and Krueger, 1987; Gilhar *et al*, 1998). Separation of $CD4^+$ and $CD8^+$ cell subsets and their transfer to this model suggested each cell type alone was not capable of reinducing hair loss, but in combination hair loss redeveloped (Gilhar *et al*, 2002). These studies suggest that the immune system and lymphocytes in particular are of primary importance in AA pathogenesis.

Using the C3H/HeJ mouse model for AA (Sundberg *et al*, 1994), we examined the potential for $CD4^+$, $CD4^+/CD25^-$, $CD8^+$, $CD19^+$ (predominantly B cell), and $CD11c^+$ (predominantly dendritic cell) cell subsets derived from AA-affected mice to transfer the disease phenotype to unaffected, immunocompetent mice. We also examined the potential for the $CD4^+/CD25^+$ candidate regulatory cell subset to blockade alopecia initiated by $CD4^+$ and $CD8^+$ cell transfer. The cell subset recipients were examined by histology and flow cytometry to characterize the injected cell host's response and to differentiate between mice that developed AA and those that did not.

Results and Discussion

As predicted by previous studies (Carroll *et al*, 2002), the comparative control groups injected with unseparated lymph node cells derived from normal haired, and AA-affected donor mice yielded no AA development and a systemic AA expression frequency of 80%, respectively (Table I). The high frequency of AA development in the group receiving cells obtained from AA-affected mice indicates that all adult C3H/HeJ mice are readily susceptible to AA development.

$CD8^+$ cells confer localized hair loss whereas $CD4^+/CD25^-$ cells promote systemic AA In this study, all 10 recipients that received subcutaneous injections of $CD8^+$ cells alone developed localized hair loss at the site of cell injection, but no hair loss elsewhere, first observed 3–5 wk after injection and persisting until necropsy at 20 wk post-injection (Table I) (Fig 1*a*). Only one $CD8^+$ cell-injected mouse developed systemic AA with hair loss beyond the cell injection site, a statistically insignificant frequency of systemic AA development ($p = 0.33$). This suggests that activated $CD8^+$ cells are capable of targeting hair follicles and are likely the primary mediators of actual hair loss in the pathogenic disease mechanism. However, in the absence of other primed immune cell subsets, $CD8^+$ cells alone are largely unable to activate the immune mechanisms required for systemic AA development beyond the cell injection site. This has implications for our understanding of how AA is perpetuated and spreads. In and of itself, hair follicle disruption by $CD8^+$ cells is apparently not enough to elicit a systemic immune response, which also implies that the AA autoantigen is not created/deliberated solely by the destruction of anagen hair follicles.

Compared with mice receiving $CD8^+$ cells alone, relatively little or no hair loss localized at the site of injection was observed after injection of $CD4^+$ or $CD4^+/CD25^-$ cells (Table I). In contrast, a single subcutaneous injection of $CD4^+$ cells promoted the development of systemic AA, that is AA beyond the immediate site of cell injection, in three of 10 (30%) naive hosts. With further purification of the cell subset to remove $CD4^+/CD25^+$ cells and isolate $CD4^+/CD25^-$ cells for injection, the frequency of systemic AA induction after injection was increased. Seven weeks after injection, 18 of 23 mice receiving $CD4^+CD25^-$ cells showed signs of systemic AA, although the alopecia patches were small. At 20 wk post-injection, eight mice that had hair loss at 7 wk had apparently recovered from the inflammatory insult and alopecia was no longer visible anywhere, but for 10 of 23 recipients (43%) the AA had become extensive and covered more than 50% of their total skin area (Fig 1*b*), a statistically significant expression frequency ($p = 0.0007$).

That there was little or no circumscribed hair loss at the site of cell injection suggests that $CD4^+$ cells do not directly contribute to inducing hair loss in the absence of other activated cell subsets. But the $CD4^+$ cell population was capable of inducing a systemic response and induction of extensive alopecia beyond the site of cell injection, demonstrating that these cells are key mediators of systemic AA pathogenesis. The systemic development of AA in the relative absence of hair follicle disruption localized to the site of cell injection also implies that the AA autoantigen is naturally presented. Further, as the majority (80%) of mice receiving $CD4^+CD25^-$ cells developed early signs of systemic AA at 7 wk (but AA was only seen in 43% at necropsy, 20 wk postinjection), we assume that early signs of AA may have been because of transferred, activated Th cells and that the amount of naturally presented autoantigen may not have been sufficient in all mice to fully reactivate memory T cells. Of eight mice receiving $CD4^+/CD25^-$ cells in combination with $CD8^+$ cells (Table I), five developed alopecia localized at the site of injection and these same mice subsequently developed systemic AA beyond the injection site that persisted until necropsy. The frequency of systemic AA development was statistically significantly above that observed with injection of $CD4^+/CD25^-$ cells alone ($p = 0.004$). The increased frequency of persistent, systemic AA development with a combined injection of $CD4^+/CD25^-$ and $CD8^+$ cells suggests a synergistic activity of these cells types in AA pathogenesis.

$CD4^+$ T cells are capable of interaction with major histocompatibility complex (MHC) class II-expressing antigen-presenting cells (APC), leading to production of interleukin (IL)-2 and enhancement of APC ability to activate other immune system components, particularly $CD8^+$ cells, through presentation of antigen in context with MHC class I (Ridge *et al*, 1998; Schoenberger *et al*, 1998; Belz *et al*, 2002). The enhancement of APC activity by $CD4^+$ cells is predominantly mediated by CD40–CD40L interaction and is characterized by upregulation of co-stimulatory receptors CD80/CD86 and IL-12 production (Cella *et al*, 1996; Grewal *et al*, 1996). Previously, flow cytometry and microarray studies demonstrated AA developed in association with increased expression of all these factors (Carroll *et al*, 2002; McElwee *et al*, 2002; Zöller *et al*, 2002). The interaction of a host APC with injected, AA antigen-specific $CD4^+$ T cells may stimulate naive, endogenous $CD8^+$ cytotoxic T cells,

Table I. Alopecia areata (AA) development with transferred cell type

Group	Cell type (number injected)	Mice with alopecia localized at the injection site	Mice with systemic AA expression after 20 wk	Confidence interval (according to Wilson)	Systemic AA expression p-value (compared with 1, non-AA)	Systemic AA expression p-value (compared with 1a, AA)	Systemic AA expression p-value (group:group comparison)
1	LNC non-AA[a] (1×10^7)	0/20 (0%)	0/20 (0%)	(0%–10%)			
1a	LNC AA[b] (1×10^7)	8/10 (80%)	8/10 (80%)	(49%–94%)	<0.0001		
2	$CD8^+$ (1×10^7)	10/10 (100%)	1/10 (10%)	(0.5%–40%)	0.33	0.006	
3	$CD4^+$ (1×10^7)	0/10 (0%)	3/10 (30%)	(11%–60%)	0.03	0.07	
4	$CD4^+/CD25^-$ (2×10^7)	4/23 (17%)	10/23 (43%)	(26%–63%)	0.0007	0.07	
5	$CD4^+/CD25^+$ (1×10^7)	0/12 (0%)	2/12 (17%)	(5%–45%)	0.13	0.008	
6	$CD11c^+$ (1×10^7)	0/9 (0%)	0/9 (0%)	(0%–30%)	1.00	0.0007	
7	$CD19^+$ (2×10^7)	0/8 (0%)	0/8 (0%)	(0%–30%)	1.00	0.001	
8	$CD8^+$ (2×10^7), $CD4^+/CD25^+$ (1×10^7)	5/5 (100%)	0/5 (0%)	(0%–43%)			(2:8) 1.00
9	$CD4^+/CD25^-$ (2×10^7), $CD4^+/CD25^+$ (1×10^7)	0/10 (0%)	0/10 (0%)	(0%–28%)			(4:9) 0.0148
10	$CD4^+/CD25^-$ (2×10^7), $CD8^+$ (1×10^7)	5/8 (63%)	5/8 (63%)	(31%–86%)			(2:10) 0.0040
							(4:10) 0.40

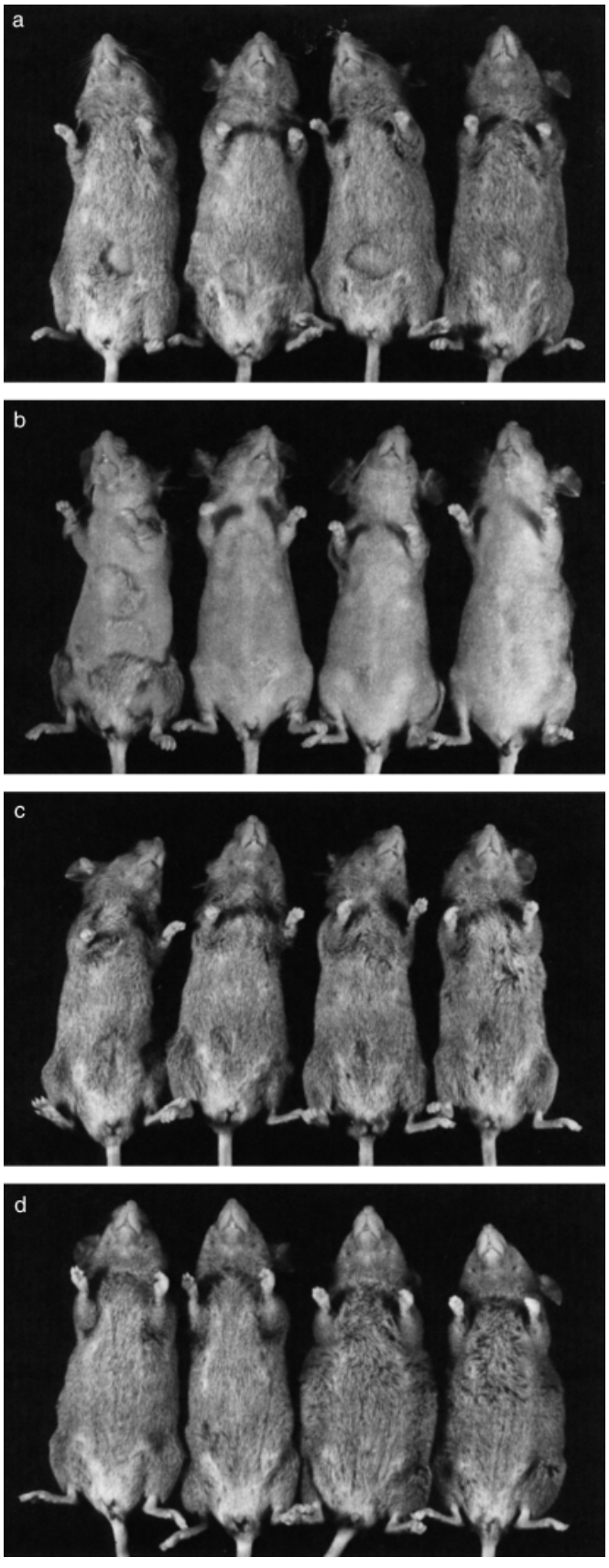
[a]Mice injected with LNC from normal haired donors.
[b]Mice injected with LNC from AA-affected donors.

which may then function as specific effector cells and target hair follicles. Much the same mechanism has been shown to occur in tumor targeting by the immune system. Transfer of tumor antigen-specific $CD4^+$ cells successfully elicits a $CD8^+$ cell targeting of the tumor via APC activation (Surman *et al*, 2000).

$CD19^+$ and $CD11c^+$ cells fail to promote hair loss Transfer of $CD19^+$ cells, predominantly a marker for B cells, and CD11c primarily expressed on dendritic cells, candidate APC, each failed to transfer a localized or systemic AA phenotype. Histology and immunohistology of $CD19^+$ and $CD11c^+$ cell recipient mice failed to demonstrate any significant differential activity in the skin as compared with controls (data not shown). For $CD11c^+$ cells, this may seem surprising. The most likely explanation would be that there were too few AA autoantigen-loaded APC in the draining lymph nodes that were used for the transfer. Alternatively, the APC derived from the draining LNC no longer displayed a migratory phenotype. That $CD19^+$ B cells were unable to transfer AA is less surprising. Although production of hair follicle-specific antibodies by B cells can be identified in the C3H/HeJ mouse model (Tobin *et al*, 1997), this production is likely to be secondary to a primary, cell-mediated perturbation of hair follicles (Carroll *et al*, 2002). This does not, however, render B cells and antibody production irrelevant to the progression of AA. Autoantibodies may have a secondary role in disease exacerbation and activated B cells can play an APC role (Serreze *et al*, 1998).

$CD4^+/CD25^+$ cells can regulate AA Transfer of $CD4^+/CD25^+$ cells has been shown to prevent autoimmune disease in several models (Sakaguchi *et al*, 1995; Suri-Payer *et al*, 1998) and we sought to examine the potential of $CD4^+/CD25^+$ cells to modulate AA development. The depressed frequency of $CD4^+/CD25^+$ cells in AA-affected mice (Zöller *et al*, 2002) significantly limited the availability of cells for use in AA transfer studies. However, of the 12 mice injected with $CD4^+/CD25^+$ cells alone, 10 failed to develop localized or systemic AA over 20 wk until necropsy. Two did develop systemic AA very late at 18 wk post-injection. Although cells with constituent $CD4^+/CD25^+$ expression are purportedly regulatory cells, it is likely there are some $CD4^+$ pathogenic cells with an induced $CD25^+$ expression within the $CD4^+/CD25^+$ population derived from mice with overt AA. This may account for the transfer of AA in two mice. Overall, however, the relative lack of AA induction and the late induction of hair loss in those mice that did develop overt AA is consistent with the hypothesis that $CD4^+/CD25^+$ cells are candidate regulatory cells. Most significantly, co-transfer of $CD4^+/CD25^+$ cells with $CD4^+/CD25^-$ cells or $CD8^+$ cells apparently prohibited the hair loss patterns seen with these pathogenic cell subsets when injected alone (Fig 1*c*, *d*). All five mice injected with $CD8^+$ and $CD4^+/CD25^+$ cells failed to present large areas of hair loss at the injection site, nor did they develop systemic AA. Similarly, none of the 10 mice injected with $CD4^+/CD25^+$ cells plus $CD4^+/CD25^-$ cells developed overt alopecia, a result that approached statistical significance ($p = 0.0148$). Boosting the activity or increasing the numbers of $CD4^+/CD25^+$ cells has been suggested as one approach in the

treatment of chronic inflammatory disease (Horwitz *et al*, 2002; Lee *et al*, 2003). Although still very much a theoretical option, the studies here suggest that the promotion of $CD4^+/CD25^+$ cells may be beneficial overall to treating AA.



Alopecia development after cell injection is associated with increased skin lymphocyte cell infiltration Histology and immunohistology of $CD8^+$ cell-injected mice revealed a local infiltrate at the site of injection largely comprised of $CD8^+$ cells (not shown). The $CD8^+$ cells had a focal peri- and intra-follicular infiltration presentation. Affected hair follicles were in dystrophic anagen or in telogen. In $CD8^+$ cell-injected mice, the skin beyond the injection site presented with a low level $CD4^+$ and $CD8^+$ cell infiltration. While the lymphocytes were hair follicle focused, the cells' position was exclusively peri-follicular. Immunohistology of mice receiving $CD4^+$ cells and developing alopecia revealed a focal follicular infiltrate of lymphocytes typical of that observed in spontaneous and skin graft-induced mouse AA (McElwee *et al*, 1998, 2002; Zöller *et al*, 2002) with $CD4^+$ cells exclusively in a peri-follicular location and $CD8^+$ cells in both peri- and intra-follicular locations (not shown).

Quantification of the number of cells extracted from the skin at necropsy prior to flow cytometry analysis demonstrated that all cell-injected mice had more cells extracted as compared with control, non-injected mice regardless of whether the mice developed AA (Fig 2*a*). Comparison of mice that developed alopecia with those that failed to develop AA within respective mouse groups injected with the same cell type, however, showed that greater numbers of cells were retrieved from those with overt hair loss. An increased number of draining LNC were only recovered from mice that developed AA (Fig 2*b*). Interestingly, all mice that developed AA displayed an increased percentage of $CD4^+$ skin-infiltrating leukocytes (SkIL), independent of whether the mice received $CD4^+$ cell injections (Fig 2*c*). In fact, the highest percentage of $CD4^+$ cells was seen in mice developing localized AA after the transfer of $CD8^+$ cells. It is possible that through hair follicle destruction a strong, but AA-independent, inflammatory response was induced. The same phenomenon, although less pronounced, may account for the percentage of $CD8^+$ cells. No increase in the percentage of $CD4^+$ or $CD8^+$ cells was seen in mice that did not develop AA (Fig 2*c*). In mice developing AA, there was a decrease in the percentage of B cells and of monocytes (data not shown). Considering the increased number of recovered SkIL in AA mice and the increased percentage of $CD4^+$ and $CD8^+$ cells within the recovered SkIL population, the reduction in B cells and monocytes appeared to be relative.

Alopecia development is associated with increased CD44 variant, pro-inflammatory cytokine, and co-stimulatory ligand activation but localized alopecia is associated with depressed co-stimulatory ligand expression CD44 variants, particularly CD44v3 and CD44v10,

Figure 1
Localized and systemic hair loss with injection of $CD8^+$ and $CD4^+$ cell combinations. Twenty weeks after injection of $CD8^+$ cells, recipient mice exhibited localized hair loss only at the site of injection (*a*). In contrast, injection of $CD4^+/CD25^-$ cells alone resulted in minimal alopecia at the site of injection, but systemic alopecia developed in a subset and was extensive by 20 wk postinjection (*b*). Combined injection of $CD8^+$ cells plus $CD4^+/CD25^+$ cells yielded localized hair loss but to a much more limited extent (*c*) whereas combination of $CD4^+/CD25^-$ plus $CD4^+/CD25^+$ cells resulted in no overt hair loss induction (*d*).

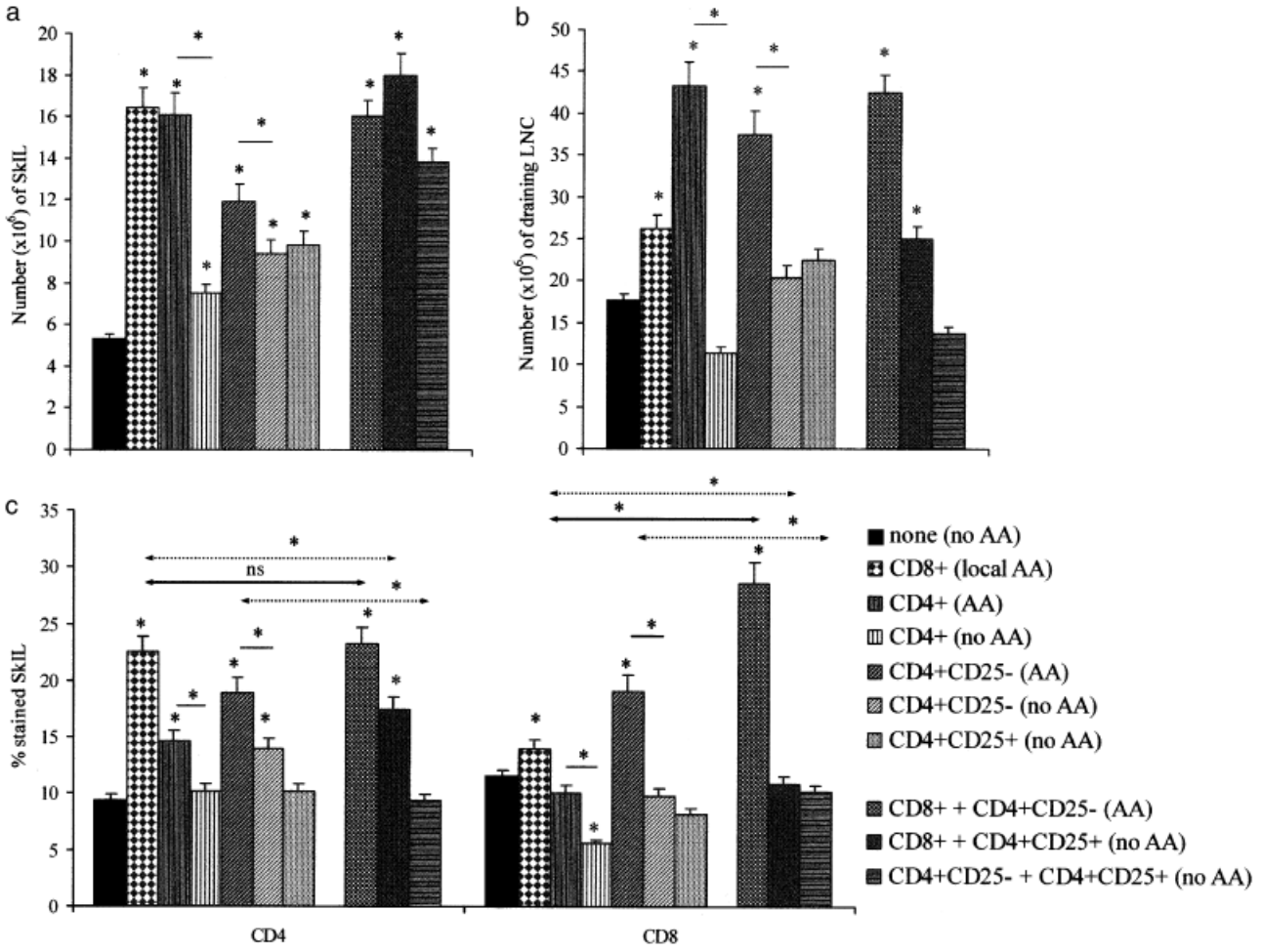


Figure 2
Increased cell numbers extracted from alopecia areata (AA)-affected mice and increase in the percentage of $CD4^+$ and $CD8^+$ lymphocytes. Although all cell-injected mice had greater numbers of skin-infiltrating leukocytes extracted from their skin compared with non-injected mice, leukocytes were retrieved in greater numbers from mice that developed localized or systemic alopecia as compared with mice that failed to develop alopecia (*a*). In contrast, a strongly increased number of draining LNC was only recovered from mice that developed systemic AA (*b*). Furthermore, a significant increase in the percentage of $CD4^+$ and $CD8^+$ skin-infiltrating leukocytes (SkIL) was seen in all mice developing localized or systemic AA, but not in mice that did not develop AA. When mice received $CD8^+$ or $CD4^+/CD25^-$ plus $CD4^+/CD25^+$ cells, the relative percentage of $CD4^+$ cells was significantly decreased (*c*). Mean + SD values of three independently performed assays are shown. Significant differences ($p < 0.01$) are indicated by an asterisk.

have been described as skin-homing receptors (Rösel *et al*, 1997; Seiter *et al*, 1999). The percentage of pan$CD44^+$ SkIL was increased in mice developing localized or systemic AA, primarily because of CD44v3 and CD44v10 expression, consistent with the supposed skin-homing function of these CD44 variant isoforms. The activation marker CD44v6 was upregulated primarily in mice receiving $CD4^+$ cells, irrespective of whether or not they developed AA, and CD44v7 expression was only increased in mice that received $CD4^+$ cells and developed AA (Fig 3). Although the latter finding is in line with apoptosis resistance induction by CD44v7 (Marhaba *et al*, 2003 and see below), the strong upregulation of CD44v6 in mice receiving $CD4^+$ cells, but not developing AA, indicates that cell transfer-induced AA development may also be determined by regulatory phenomena.

Cytokines have repeatedly been described as contributing to AA development, although no clear Th1 or Th2 profile has emerged (Hoffmann, 1999; McElwee *et al*, 2002). After T cell transfer, there was a strong upregulation of IL-6, tumor necrosis factor (TNF)α, IL-12, and interferon (IFN)γ expression in mice developing localized or systemic AA. These indicators reconfirm the activated status of SkIL. But IL-6 and IFNγ expression were also increased, although to a minor degree, in mice receiving $CD4^+/CD25^+$ cells that did not develop AA. These mice also displayed very high-level expression of IL-10. $CD4^+/CD25^+$ cells are known largely not to secrete cytokines with the exception of IL-10 and transforming growth factor β at a low level (Shevach, 2002; Nakamura *et al*, 2004), however, they can infectiously induce IL-10 expression (Jonuleit *et al*, 2002; Stassen *et al*, 2004). Our findings strongly suggest that the prevention of AA development after the transfer/co-transfer of $CD4^+/CD25^+$ cells at least partly relies on the high-level regulatory IL-10 cytokine expression (Fig 4).

Of cell co-stimulatory ligands, CD40, CD80, and CD86 were found with increased frequency on SkIL from mice with systemic AA, whereas mice with $CD8^+$ cell-induced localized AA presented with a slightly reduced expression frequency for CD40 and CD86, and significantly so for CD80 (Fig 5). This failure in co-stimulatory ligand expression may

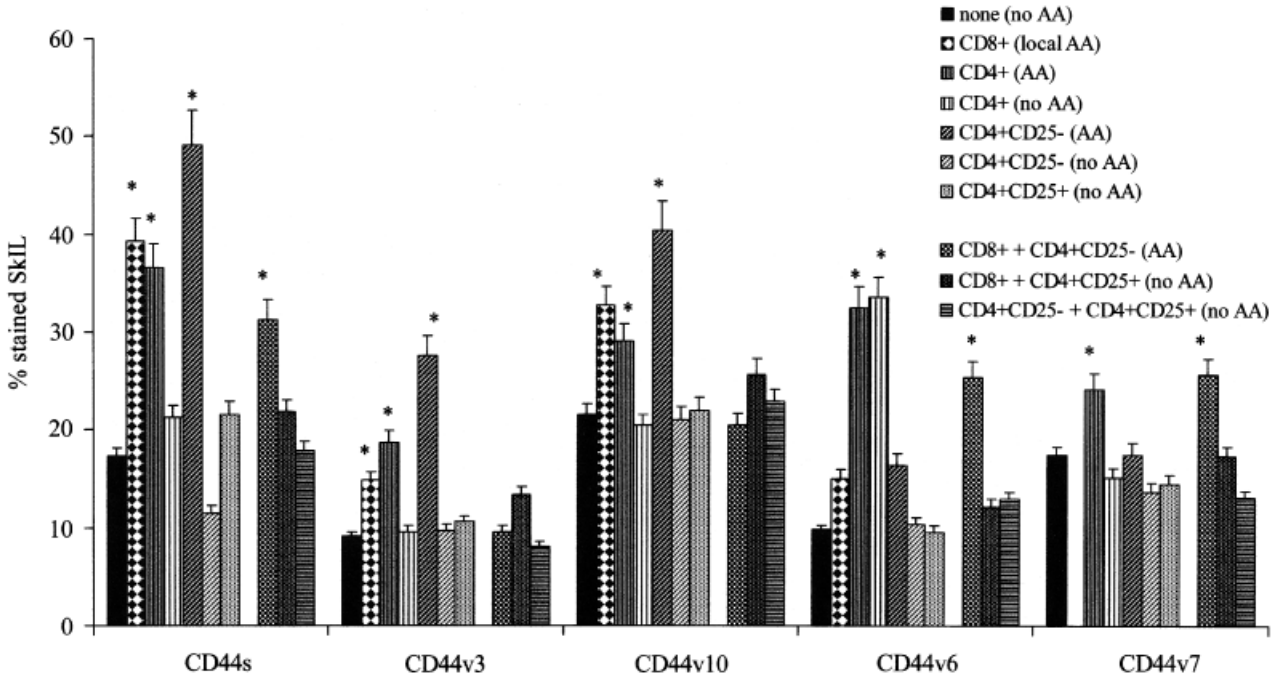


Figure 3
Skin-homing receptors are expressed with increased frequency on skin-infiltrating leukocytes from alopecia-affected mice. Mice with localized or systemic alopecia development revealed skin-infiltrating leukocyte populations with increased expression of skin-homing receptors CD44v3 and CD44v10. The activation marker CD44v6 was particularly high after the transfer of $CD4^+$ cells and independent of systemic alopecia areata (AA) induction. Upregulation of CD44v7 expression was only seen in AA mice after the transfer of $CD4^+$ cells. Mean + SD values of three independently performed assays are shown. Significant differences ($p < 0.01$) are indicated by an asterisk.

be one explanation for the lack of systemic AA development despite the apparent increased presence of $CD4^+$ cells in the SkIL population after $CD8^+$ cell injection. The dynamics of this successful localized alopecia induction, and its relative failure to induce systemic AA, is not defined in this study. In other autoimmune disease models, however, $CD8^+$ cells have been defined as both regulators (Kadowaki *et al*, 1994) and promoters (Huseby *et al*, 2001) of disease pathogenesis suggesting potential functional heterogeneity in this population. It is possible that there are $CD8^+$ regulatory cells among the injected cell population. Although activated, pathogenic $CD8^+$ cells may promote alopecia localized at the site of injection, it is possible that a second regulatory $CD8^+$ cell population may be actively involved in systemic damage limitation. Notably, too, CD40, CD80, and CD86 expression was upregulated in mice receiving $CD8^+$ or $CD4^+/CD25^-$ cells together with $CD4^+/CD25^+$ cells, although these mice did not develop AA. In addition to evidence of IL-10 secretion, this is more evidence that the failure to develop AA may not be solely because of a lack of activation, but may also be sustained by a counter-regulation of response.

$CD4^+/CD25^+/CD152^+$ regulatory cells are depressed in numbers relative to pathogenic $CD4^+/CD25^+/CD154^+$ cells in mice that developed AA The importance of regulatory phenomena was further supported by the exploration of the ratio of regulatory *versus* activated Th cells in the skin of transferred mice. Examination of the $CD4^+/CD25^+$ population for an activated ($CD154^+$, also named CD40L) *versus* regulatory ($CD152^+$, also named CTLA4) phenotype demonstrated a greater ratio of $CD4^+/CD25^+/CD154^+$ cells to $CD4^+/CD25^+/CD152^+$ cells in mice that developed AA. In direct contrast, mice with failed alopecia induction presented with a greater ratio of regulatory phenotype $CD4^+/CD25^+/CD152^+$ cells. These patterns disappeared after *in vitro* stimulation (Fig 6). The presence of $CD154^+$ cells in the $CD4^+/CD25^+$ cell population provides further evidence in support of the explanation as to why two mice developed AA after receiving $CD4^+/CD25^+$ cells. Although this population is strongly enriched for cells with a regulatory phenotype, some cells may be recently activated, pathogenic cells. Overall, however, the regulatory cell phenotype defined by $CD4^+/CD25^+$ expression is important in AA susceptibility. While injection of these cells can help prevent AA transfer, their loss in the initiation of AA may be a significant factor in disease perpetuation.

AA development is associated with an increase in leukocyte cell resistance to apoptosis In addition to immunosuppressive cytokines and regulatory T cells, activation-induced cell death (AICD) is the third regulatory element by which undue activation of the immune system is avoided (Lenardo *et al*, 1999). Autoimmune diseases are frequently associated with defects in pro-apoptotic molecules or overexpression of anti-apoptotic molecules. We asked whether apoptosis susceptibility and expression of pro-apoptotic molecules might correlate with cell transfer-induced AA. Apoptosis susceptibility was evaluated by Annexin V staining. Annexin V has high affinity for phosphatidylserine, which is normally only expressed on the inner plasma membrane leaflet of healthy cells, but becomes externalized in early apoptosis (Martin *et al*, 1995; Vermes *et al*, 1995). Annexin V binding was reduced in SkIL from

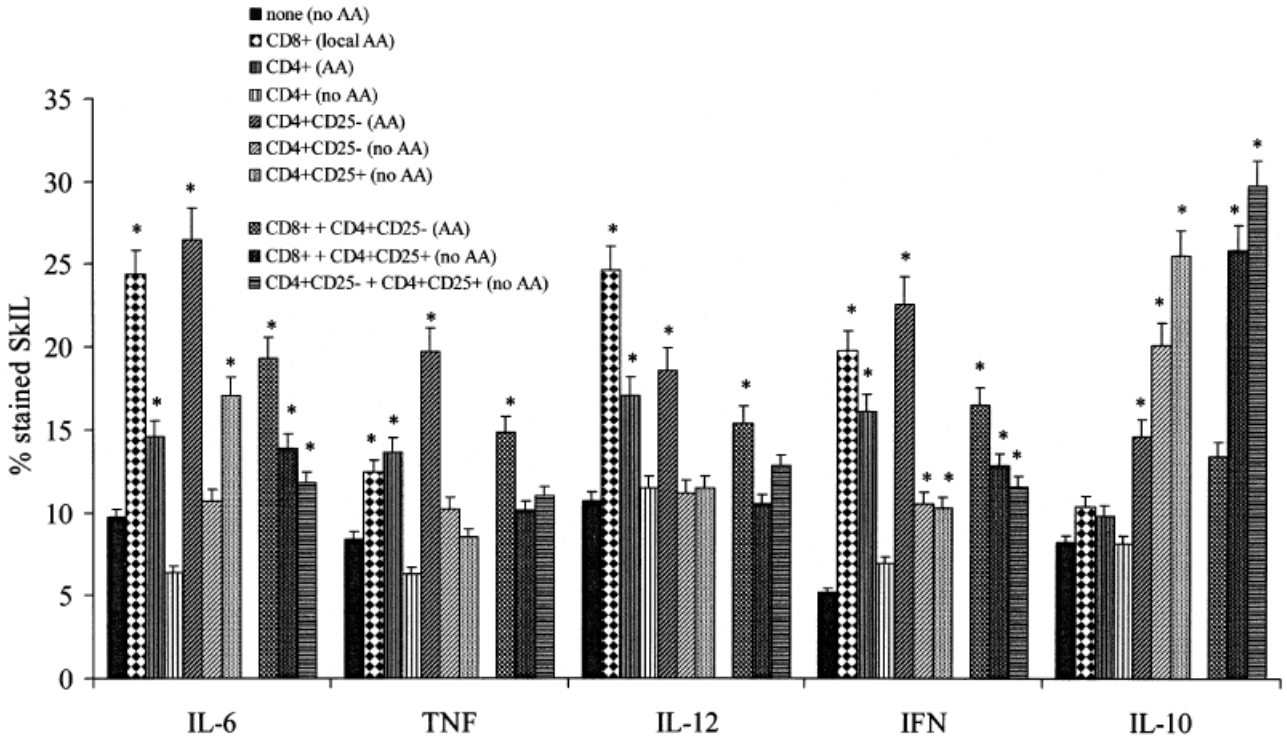


Figure 4
Increased pro-inflammatory cytokine expression in skin-infiltrating leukocytes with alopecia development. Mice with alopecia development exhibited increased frequencies of inflammatory leukocytes expressing interleukin (IL)-12, interferon (IFN)γ, IL-6, and tumor necrosis factor (TNF)α. With the exception of mice receiving $CD4^+$ cells, IL-10 expression was high in mice not developing alopecia areata (AA) and was further increased in mice receiving mixtures of lymphocytes including $CD4^+/CD25^+$ cells. Mean + SD values of three independently performed assays are shown. Significant differences ($p < 0.01$) are indicated by an asterisk.

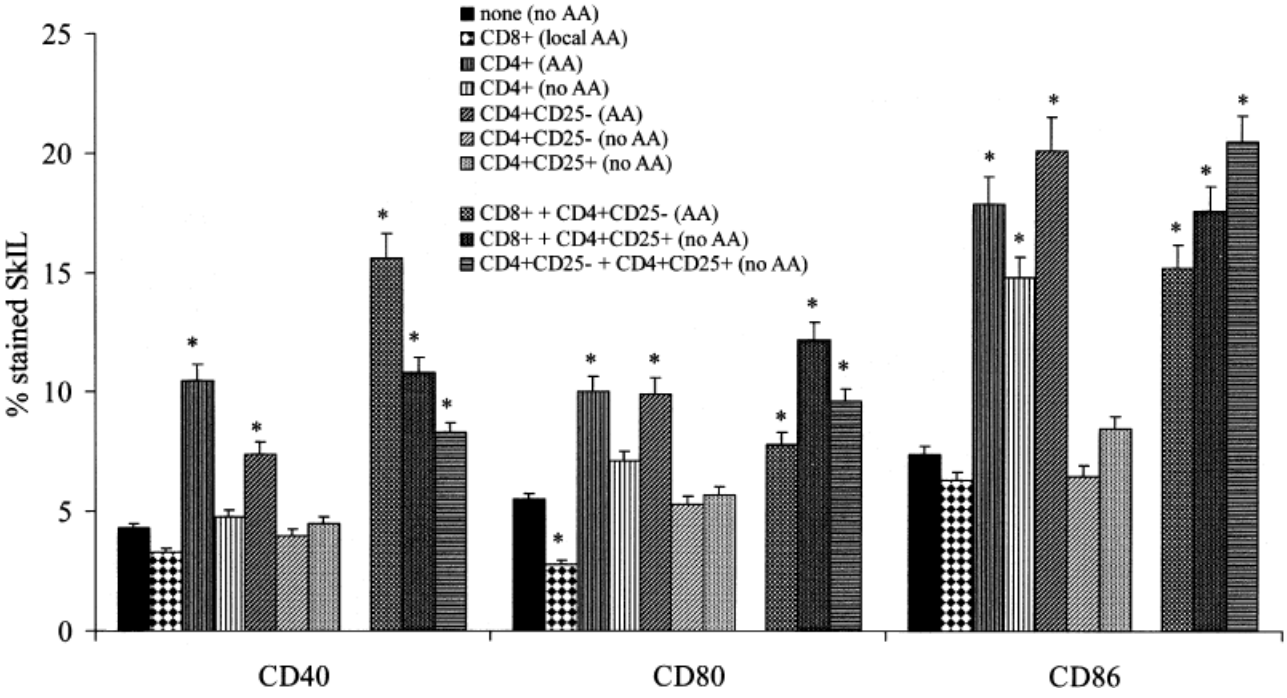


Figure 5
Alopecia development is associated with upregulation of costimulatory ligands. The skin-infiltrating leukocyte population from systemic alopecia-affected mice contained more cells with CD40, CD80, and CD86 expression. But costimulatory molecule expression was also high in mice that received mixtures of $CD8^+$ or $CD4^+/CD25^-$ and $CD4^+/CD25^+$ cells. Leukocytes from CD8+ cell-injected mice presented with reduced expression, particularly of CD80. Mean + SD values of three independently performed assays are shown. Significant differences ($p < 0.01$) are indicated by an asterisk.

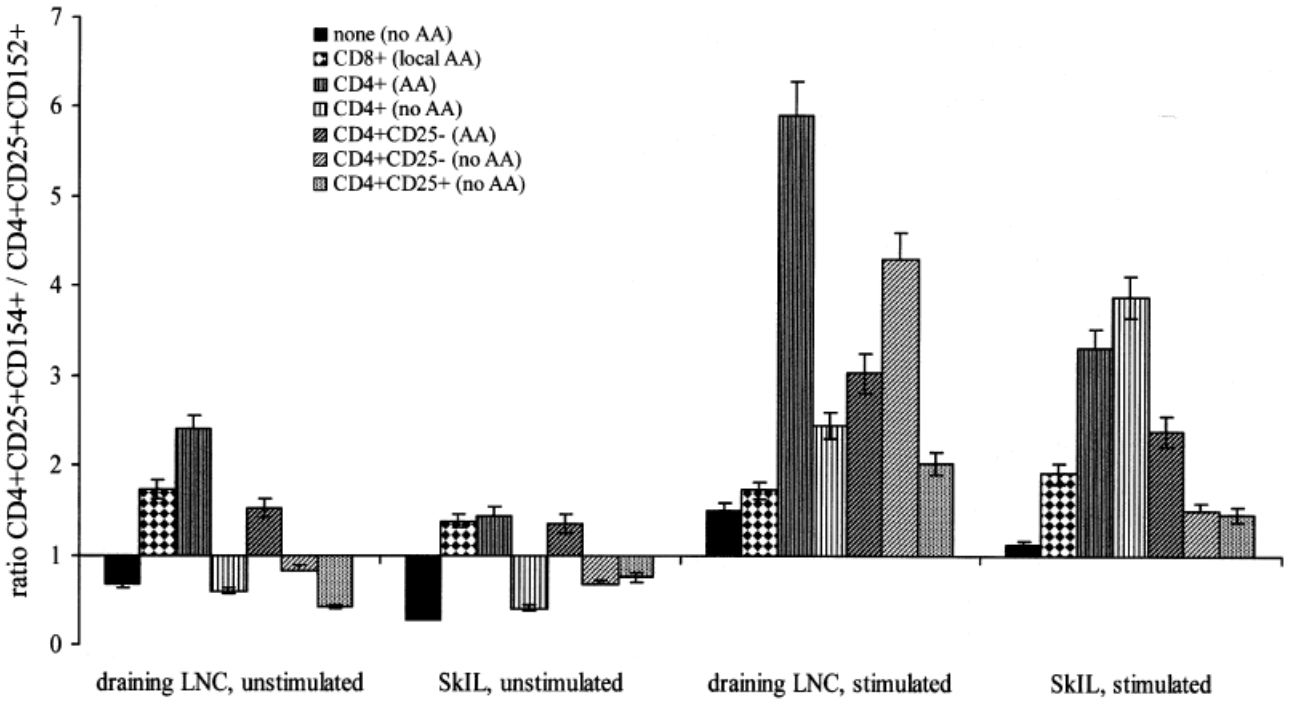


Figure 6
Alopecia areata (AA) is associated with greater numbers of pathogenic $CD4^+/CD25^+/CD154^+$ cells. The ratio of candidate pathogenic $CD4^+/CD25^+/CD154^+$ cells to regulatory $CD4^+/CD25^+/CD152^+$ cells in both draining lymph nodes and skin was greater in AA-affected mice with an up to twofold difference. Stimulation abrogated the distinction. Mean ± SD of three independently performed assays is shown.

AA-affected mice indicating reduced responsiveness to apoptosis-inducing mechanisms and cellular resistance to apoptosis in the SkIL population (Fig 7). Annexin V staining was also reduced, although less pronounced, in mice receiving $CD4^+/CD25^-$ plus $CD4^+/CD25^+$ cells, indicating that AA resistance in these mice may not be because of resistance towards AICD.

CD95 (Fas) and CD95L (FasL) and liganding of CD120a (TNF receptor 1) and CD120b (TNF receptor 2) via TNFα or LTα/LTβ binding, respectively (Wallach *et al*, 1999; Schmitz *et al*, 2000), are the most prominent death-inducing receptor/ligand pairs. CD95L in particular is constitutively expressed at a higher level in SkIL than other lymphoid organs.[1] Examination of SkIL for apoptosis promoting markers revealed significantly decreased expression of CD95, CD95L, CD120a, and CD120b in mice that developed AA. Notably, CD120a and CD95L expression were upregulated in mice that did not develop AA after receiving $CD4^+/CD25^-$ cells (Fig 7). This apparent reduced leukocyte susceptibility to AICD via reduced CD95–CD95L and CD120 expression may additionally support AA perpetuation. This may explain the observed response to the transfer of $CD4^+/CD25^-$ cells in particular, as in these mice, not only was downregulation of death receptors seen in AA but also upregulation of death receptors in mice that did not develop AA.

Summary Although *in vitro* activation of cells prior to transfer is sometimes required in other autoimmune disease models (Braley-Mullen *et al*, 1985; Clark and Lingenheld, 1998; Gilhar *et al*, 1998), in this study *in vitro* stimulation was not necessary and the cells retained their apparent *in vivo* conditioned properties throughout the cell separation process. Not using *in vitro* stimulation avoids promoting proliferation of cell clones that may not be fundamentally involved in the *in vivo* disease state. The study also demonstrates that normal mouse pelage hair follicles, not previously exposed to AA mechanisms, can be targeted by *in vivo* primed immune cells. The $CD4^+/CD25^-$ cell population in the C3H/HeJ mouse model retains the capacity to transfer the systemic AA phenotype to naive hosts and most likely promotes AA via promotion of APC activity and subsequent stimulation of hair follicle autoreactive $CD8^+$ cells. This pathogenic activity defines the $CD4^+/CD25^-$ cell population as a key component of AA and a prime target for the development of therapeutic strategies. Understanding the mechanisms of which $CD4^+/CD25^-$ cells interact with APC in AA and which specific $CD4^+/CD25^-$ cell clones are truly pathogenic will provide new insights into AA pathogenesis. The $CD4^+/CD25^+$ cell population has been confirmed as functionally a predominantly regulatory cell subset in AA. Boosting regulatory cell activity may be one method of counteracting $CD4^+/CD25^-$ pathogenicity.

Materials and Methods

Mice All procedures were approved by the regional animal ethics committee and in accordance with state guidelines. C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) received autoclaved food pellets (altromin 1434, Altromin GmBH, Lage, Germany) and acidified water (pH 2.8–3.0) *ad libitum*. Twenty normal haired female mice (control) and 150 + female, AA-affected mice, produced by grafting skin from spontaneously AA-affected mice to normal haired recipients as previously described (McElwee *et al*, 1998), were used as cell donors. All recipients were females and allotted to age-matched groups of 10–20 wk.

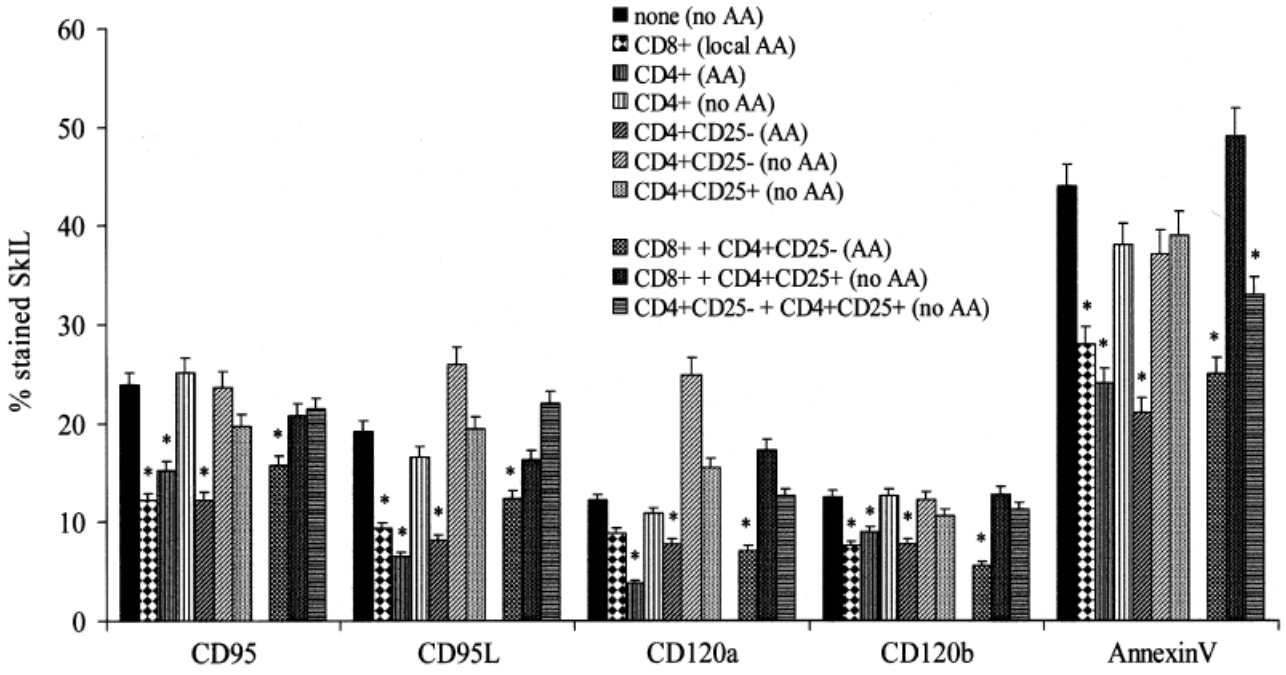


Figure 7
Increased leukocyte resistance to apoptosis in alopecia areata (AA)-affected mice. Skin-infiltrating leukocytes from mice with either localized or systemic alopecia demonstrated reduced expression of CD95, CD95L, CD120a, and CD120b. This reduction in receptors associated with activation-induced cell death was associated with reduced annexin V binding, an indicator of apoptosis susceptibility. Mean + SD values of three independently performed assays are shown. Significant differences ($p < 0.01$) are indicated by an asterisk.

Cell transfer Normal haired and AA-affected C3H/HeJ donor mice were euthanized by cervical dislocation. Single cell suspensions were prepared from pooled skin draining lymph nodes (axillary, brachial, superficial cervical, inguinal) or, in the case of $CD19^+$ cells, spleens of AA-affected mice. For comparative controls, 20 mice were injected subcutaneously with unseparated lymph node cells from normal haired donors and 10 mice received equivalent cell injections from AA-affected mice. Cell subsets were isolated using antibody-conjugated magnetic particle separation (Radbruch *et al*, 1994). Cells were selected ready for transfer-using combinations of anti-CD19 (clone 1D3), CD4 (clone GK1.5), CD8a (clone 53–6.7), and CD11c (clone N418) mAb (All Miltenyi, Bergisch Gladbach, Germany) and manufacturer's standard protocols. $CD4^+/25^+$ and $CD4^+/CD25^-$ cells were isolated by non-T cell depletion followed by anti-CD25-phycoerythrin (PE) (clone 7D4) and anti-PE magnetic particles. Positive selection does not induce activation in the selected cells (Nohe *et al*, 2002). Recipients' injected cell combinations and cell numbers are listed in Table I. Confidence intervals for systemic AA expression in the different mouse groups were calculated according to Wilson's method (Newcombe, 1998). Statistical analyses of systemic AA frequencies in different groups were performed with Fisher's exact test (Agresti *et al*, 1990).

Flow cytometry For *in vitro* stimulation assays and flow cytometry analyses, the entire dorsal and ventral skin and draining lymph nodes were collected at necropsy 20 wk after cell injection. Single leukocyte cell suspensions were prepared from the skin as previously described (Zöller *et al*, 2002). SkIL from two to three animals were pooled. Viability was determined by Trypan blue exclusion and was in the range of 70%–80% in SkIL preparations. Each cell sample was aliquoted and incubated with 10 μg per mL of the primary antibody and subsequently with the appropriate anti-mouse or anti-rat PE-labeled secondary antibody, or after incubating with biotinylated mAb, with streptavidin PE. Negative controls were incubated with a non-binding primary antibody and the same secondary reagents. For intra-cellular labeling of cytokines, cells were fixed and permeabilized in advance. For double fluorescence analysis, cells were first labeled with a biotinylated antibody and Streptavidin PE followed by exposure to the second, fluorescein isothiocyanate (FITC)-labeled antibody. Annexin V (1 μg per mL) and propidium iodide (PI) (1 μg per mL) staining was performed in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Flow cytometry (FACSstar, Becton Dickinson, Heidelberg, Germany) followed routine procedures (Zöller *et al*, 2002). Cell viability was assessed by PI exclusion and cell debris and dead cells were excluded from analysis. All experiments were repeated at least three times. Values represent the mean and, where indicated, the standard deviation (SD). The significance of differences was evaluated using the two-tailed Student's *T* test.

Antibodies The following mAb were used for flow cytometry: biotinylated anti-mouse IL-6 (clone MP5-32C11), IL-10 (clone JES3-16E3), IL-12 (clone C17.8), IFNγ (clone XMG1.2), TNFα (clone MP6-XT3); unconjugated or FITC- or PE-conjugated anti-mouse CD25 (clone PC61), CD40 (clone 3/23), CD80 (clone 16-10A1), CD86 (clone GL1), CD152 (CTLA-4, clone 1B8), CD154 (CD40L, clone MR1), and sIgM (clone R6-60.2); anti-CD95 (clone Jo2), CD95L (clone MFL3), CD120a (clone 55R-286), and CD120b (clone TR75-89) (all BD/Pharmingen, Hamburg, Germany); anti-CD44v6 (clone 9A4, BenderMed Systems, Vienna, Austria), rabbit anti-CD44v7 (Chemicon International, Temeccula, California), anti-mouse CD4 (clone YTA 3.2.1), CD8 (clone YTS 169.4.2.1), and monocyte/macrophage (clone YBM 6.6.10) (European Animal Cell Culture Collection, Porton Down, UK); anti-panCD44 (clone IM7) (American Type Culture Collection, Manassus, Virginia); unconjugated or biotinylated anti-CD44v3 (clone PTS33, Seiter *et al*, 1999), and anti-CD44v10 (clone K926, Rösel *et al*, 1997); biotinylated secondary reagents, PE- and FITC-labeled anti-rat IgG, anti-mouse IgG, anti-rabbit IgG, anti-hamster IgG, and Streptavidin (all Dianova, Hamburg, Germany).

Cell stimulation assay Cytokine and co-stimulatory molecule expression was tested by flow cytometry after overnight culture of SkIL in the presence of 1 μg per mL PHA. Apoptosis susceptibility (Annexin V staining) was evaluated after overnight culture on plates coated with subthreshold levels of anti-CD3 (1 μg per mL) that is

known to drive leukocytes into apoptosis. Cell subpopulations ($CD4^+/CD25^+/CD152^+$ or $CD4^+/CD25^+/CD154^+$) were stimulated by seeding in triplicate on anti-CD3-coated flat-bottom 24-well plates (10 μg per mL) and adding 10 μg per mL anti-CD28 to the culture medium. The number of cells was kept constant (1×10^5 cells per well), but the stimulant was titrated from 10 to 1.25 μg per mL. Cells in microtiter plates were cultured for 16–48 h. Cells were harvested, centrifuged, adjusted to $1–5 \times 10^5$/100 μL and 100 μL aliquots were transferred into 96-well plates for flow cytometry.

Histology and immunohistology At necropsy, ventral skin samples at the site of injection and dorsal skin were fixed in Fekete's acid–alcohol–formalin solution and paraffin embedded for routine histology (Relyea *et al*, 1999). Skin samples were also embedded in the OCT compound (Tissue tek, Sakura, Zoeterwoude, the Netherlands) and snap frozen in liquid nitrogen for subsequent immunohistology as described elsewhere (Freyschmidt-Paul *et al*, 2000). Primary antibodies were; anti-mouse CD4 (clone RM4-5, Pharmingen, San Diego, California) and CD8 (clone 53–6.7, Southern Biotechnology, Birmingham, Alabama). Tissue sections were counter-stained with Mayer's hematoxylin. The primary antibody was replaced with normal rat IgG for negative controls.

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Address correspondence to: Kevin J. McElwee, Division of Dermatology, University of British Columbia, 835 West 10th Avenue, Vancouver, BC, Canada. Emails: kevin@keratin.com, kmcelwee@interchange.ubc.ca

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