



*Original Research Article*

# Reversal of alopecia areata in a mouse model of human hair loss by daily dietary administration of a proprietary extract of North American ginseng (*Panax Quinquefolius*): Maintenance of hair after extract withdrawal

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Decades of study on alopecia areata have concluded that the condition results from an autoimmune attack on the hair follicle growth center (anagen). In this study, we have used C3H mice, the established model of human alopecia areata. Elderly mice (18 months), almost completely alopecic, were placed on a daily conventional diet (6gm chow/mouse) for 32 weeks (8 months), into which 80mg/mouse of CVT-E002 (a proprietary extract of North American ginseng), was homogenized. These mice began to show hair re-growth after only 2 weeks, with complete hair re-growth by week 32 at which time they were euthanized. The absolute numbers ( $\times 10^6$ ) of natural killer (NK) immune cells, and of non-NK immune cells (T/B lymphocytes) in mice consuming CVT-E002, were significantly more abundant ( $p < 0.0001$ ) in both the spleen and bone marrow than in the corresponding organs of parallel, control-diet alopecic mice. In a second group of experiments, CVT-E002 was removed from the diet at week 32 and mice were placed on the control diet for the following 14 weeks (3½ months). All these mice retained their fully re-grown coats. At that time, mice were aged 29 ½ months (2½ years), i.e., 80- 90 years of age in humans.

**Key words:** ginseng, NK cells, lymphocytes, alopecia areata, pre-clinical

## INTRODUCTION

One form of human baldness, i.e., alopecia areata, is a world-wide affliction affecting individuals of both genders, all etiologies/cultures and all ages. Between one-half to two-thirds of such cases begin after puberty in early adulthood (Price, 1991). Alopecia areata is a chronic inflammatory affliction in which there is a non-destructive, immune cell attack on the cells of the root growth center (anagen). All hair-bearing skin is potentially involved and indeed, while “areata” is restricted to hair loss in specific areas, i.e. random patches on the head, it can extend to include the loss of hair on the entire head (alopecia totalis) or even the entire body (alopecia universalis). Approximately 1 in 5 people afflicted with this condition have a family tendency toward it, implying the involvement

of a genetic factor (McDonagh and Messenger, 1996). Even environmental factors have been implicated (Jackow et al., 1998). More than 3 decades of study on the mechanisms responsible for this condition, have produced a general consensus that alopecia areata is an autoimmune disease (Kos and Conton, 2009; Gilhar, 2010; Petukhova et al., 2010). Autoimmunity itself is a complex phenomenon and to date, still remains not fully understood, thus impeding precise identification of the mechanism(s) responsible for alopecia areata.

While no treatment specifically aimed at alopecia areata has yet been developed, some success has been achieved with diphenylcyclopropenone (DPCP) (Happle et al., 1983) which, although not mutagenic, is beset with negative side

effects, not the least of which is the secondary development of other pathological conditions (Henderson and Ilchyshyn, 1995; Tosti et al., 1989). Some success has also been achieved with laser treatment, quercetin, phytoestrogens, genistein, and aromatherapy (Hay et al., 1998; McElwee et al., 2003; Wikramanayake et al., 2011; 2012).

A second type of this human baldness, seen in adult males, is alopecia androgenetica which although not the topic of the present study, is the one for which the currently marked pharmaceuticals such as minoxidil, marketed as ®Rogaine, and finasteride, marketed as ®Propecia, have been developed. These agents, however, have virtually no effect on alopecia areata.

In the present study, we have used a well-established mouse model of human alopecia areata, i.e., the C3H strain (Sundberg et al., 1994; McElwee et al., 2002; McElwee et al., 2003; Wikramanayake et al., 2011; 2012), to investigate the effect of a proprietary, standardized extract of North American ginseng on hair re-growth and maintenance. We have found that daily dietary intake of an extract of North American ginseng, resulted in hair re-growth in such alopecic mice and retention of full body hair long after removing the extract from the diet.

## MATERIALS AND METHODS

### Mice

Female, retired breeder mice, of the C3H strain at 8-10 months of age were purchased. The female gender was selected because the hormonal interference was minimal for both estrogens and androgens. However, even aged male mice continue to produce androgens, and because of this, androgenic alopecia would become a confounding factor in this study.

All mice were maintained in microisolator cages under controlled environmental conditions of optimal temperature and humidity on a 12 hour light/dark cycle in a room which itself was under laminar flow conditions. In-house veterinary and technical services in the McGill University Animal Care Facility ensured, moreover, that all edicts issued by the CCAC (Canadian Council on Animal Care) were strictly adhered to, including the regulation that all personnel involved in the handling of the mice in this study had passed the required training courses in rodent husbandry. Sentinel mice in the same room housing the cages regularly demonstrated the absence of all common mouse pathogens.

By 18 months of age, mice had lost almost all their body/face hair and were clearly alopecic. At that time, these alopecic mice became the subject of the experimental protocol described below.

### The dietary additive

CVT-E002 is a proprietary extract of North American ginseng (*Panax Quinquefolius*), produced by Afexa Life

Sciences, Inc., Edmonton, AB, Canada. CVT-E002 consists of specific polysaccharides (poly-furanosyl-pyranosyl-saccharides). Both biological and chemical standardized assays were applied together with consistent manufacturing processes to ensure that both the composition and the pharmacological activity of each lot of CVT-E002 was identical (Wang et al., 2001; Shan et al., 2007), and free of microbial contaminants. The extract is a strong immune stimulant *in vivo* and *in vitro*, and results in increased production of several lymphocyte-stimulating cytokines (Wang et al., 2001; 2004).

### Treatment

A homogenized mixture of powdered standard mouse chow (LabChow, Agribrands, Inc., Woodstock, ON, Canada) ± CVT-E002 was provided daily via the diet, as follows: Mice (aged 18 months) were fed daily 6 gm of powdered chow each ± CVT-E002 (80mg CVT-E002/6gm chow/day/mouse). Since mice were housed 2/cage, each daily provision/cage consisted of 12 gm chow ± 160mg CVT-E002. We have demonstrated more than a decade ago, that adult mice consume 6 gm of chow/24 hour period. We have, therefore, used 12 gm chow/24 hour period when 2 mice were housed together. Each morning, the specially devised container with 12gm chow was empty for both control (no CVT-E002) and experimental (CVT-E002) mice. Even if there were minor discrepancies in food consumption between the 2 mice/day, the very long periods of time (months) during which this arrangement was in place, would cancel out these minor discrepancies. Moreover, at the end of these lengthy food consumption periods, all mice - both CVT-E002-consuming and the controls - were clinically healthy by all qualitative and quantitative parameters (activity levels, body weight, grooming, drinking). Finally, the quantity of 80mg of CVT-E002, spread through 6,000mg (6 gm) of chow, was not a negatively perceived taste factor, and consumption of the CVT-E002-containing chow was identical to that of controls throughout the several months of this study.

Alopecic mice were placed on the CVT-E002-containing diet for 32 weeks (8 months), while identical, control alopecic cage mates continued to receive the same diet but without CVT-E002 during this same time period. After 32 weeks of consuming the CVT-E002-containing diet daily, these mice, now 26 months of age, together with the corresponding control-diet mice, were euthanized and their spleen, bone marrow and blood were prepared for analysis of immune cells, i.e., NK cells and non-NK, T/B lymphocytes.

A second group of alopecic, CVT-E002-consuming mice was not euthanized at 32 weeks (8 months), but rather, converted to the control diet at 32 weeks and allowed to simply live on, unmanipulated, for a further 14 weeks (3 ½ months) at which time they were euthanized as healthy, active animals, now aged 29 ½ months (18 + 8 + 3 ½ months), normal by all clinical standards, including retention of their re-grown coats, simply in the interest of concluding this study.

## NK cell content of the spleen, bone marrow and blood: Extraction, identification, enumeration

We summarize here previously published methods (Miller and Kearney, 1997; Maloney et al., 1998; Sun et al., 1999; Miller et al., 2011a, Durairaj and Miller, 2012, 2013) for extracting, preparing, identifying and enumerating lymphocytes and NK cells from the spleen, bone marrow and blood of mice. Although the methods described below are labor intensive, they are highly accurate. Fluorescence-activated cell sorting (FACS) technology, while rapid and needing only cell surface markers to identify cells, has certain limitations which prevent its use in this study. First, error is high in trying to quantify populations of cells whose numbers are very low, i.e., bone marrow-based NK cells where they represent approximately 1% of total nucleated cells therein, or in the spleen where they account for approximately 5% of total nucleated cells therein. Attempting to FACS-analyze NK cells would result in their being lost in the error of the technique, i.e., hidden in the standard deviations of much larger cell populations bearing cross-reacting or identical surface markers. For example, in the different lineages of hemopoietic and immune cells, the immature stages of the various lineages have significant marker overlap with either the immature – or the mature – stages of other lineages, including NK cells.

Described here, is a method which is accurate for identifying and recording small populations of cells such as NK cells (Miller et al., 2011a; Durairaj and Miller, 2012; 2013). Upon removal of the spleen from each mouse – both experimental and control – it was pressed through a stainless steel mesh into a combination medium consisting of ice cold Eagles basal essential medium (GIBCO Invitrogen Corp., Burlington, Ontario, Canada), with 10% heat-inactivated newborn bovine serum (NBS), also sourced from GIBCO. The bone marrow was removed from both femurs from the same mice, after snipping off both ends of each femoral shaft and repeatedly flushing the bone marrow plug out using an appropriately sized needle placed on a syringe pre-filled with 1 ml of medium (above). Subsequently, for both the spleen and bone marrow, free cell suspensions were obtained by gentle, repeated pipetting. Suspensions were then layered for 7 min onto 1.5 ml pure NBS for the purpose of allowing, during this time, the sedimentation of any non-cellular (heavy) debris into the NBS. The supernatants, now containing single, free hemopoietic and immune cells, were then removed and centrifuged for 7 min (1100rpm, 4 C) and the resulting pellet was re-suspended in a fixed volume of fresh medium + NBS. The total number of nucleated cells (hemopoietic and immune cells) was then obtained for the spleen and bone marrow (2 femurs) for each mouse (CVT-E002-consuming and control) by means of a hemocytometer (American Optical Co., Buffalo, NY, USA).

After the total number of all nucleated cells was obtained for each spleen and for both femurs for every mouse, smears were made from these cell suspensions, onto Superfrost Plus® microscope slides (Fisher Scientific,

Whitby, ON, Canada). However, just prior to euthanasia for spleen and bone marrow extraction (above), blood was extracted from the same mice from the lateral tail vein and smears were directly prepared from the fresh blood.

All smears (spleen, bone marrow and blood) were then stained using MacNeal's tetrachrome hematologic stain (Sigma-Aldrich, Oakville, ON, Canada), ideally suited for the morphological identification of NK cells and all other hemopoietic and immune cells. This stain not only provides a 4-color discrimination of the key cellular components (DNA, RNA, protein), but also permits a clear distinction of the size of cells, their shape and the unique anatomical components, i.e., cytoplasmic granules, Golgi zone, stage of mitosis, etc. Each smear was then "read", i.e., a field-by-field enumeration of every cell contained in each microscopic field (x 100) was made from beginning to tail end of every smear until 1000 total spleen and blood cells and 2000 bone marrow cells were counted, and identified.

By reading the smears, the proportions (%) of NK cells could be recorded and identified distinct from all the other cell types in each smear. NK cells were identified by virtue of their being small to medium sized cells (7-9 microns in nuclear diameter), of lymphocytic morphology, but bearing 3-5 purple-stained, cytoplasmic granules (Babcock et al., 1983; Ames et al., 1989; Saksela et al., 1982), clearly visible at x100 with the light microscope. In fact, the presence of cytoplasmic granules was one of the first methods of identifying NK cells (Saksela et al., 1982). Other immune cells, i.e., T/B lymphocytes, are morphologically identical to NK cells, but never bear granules. The histology/morphology of all these immune cell types was established decades ago and can be seen in immunology atlases and textbooks.

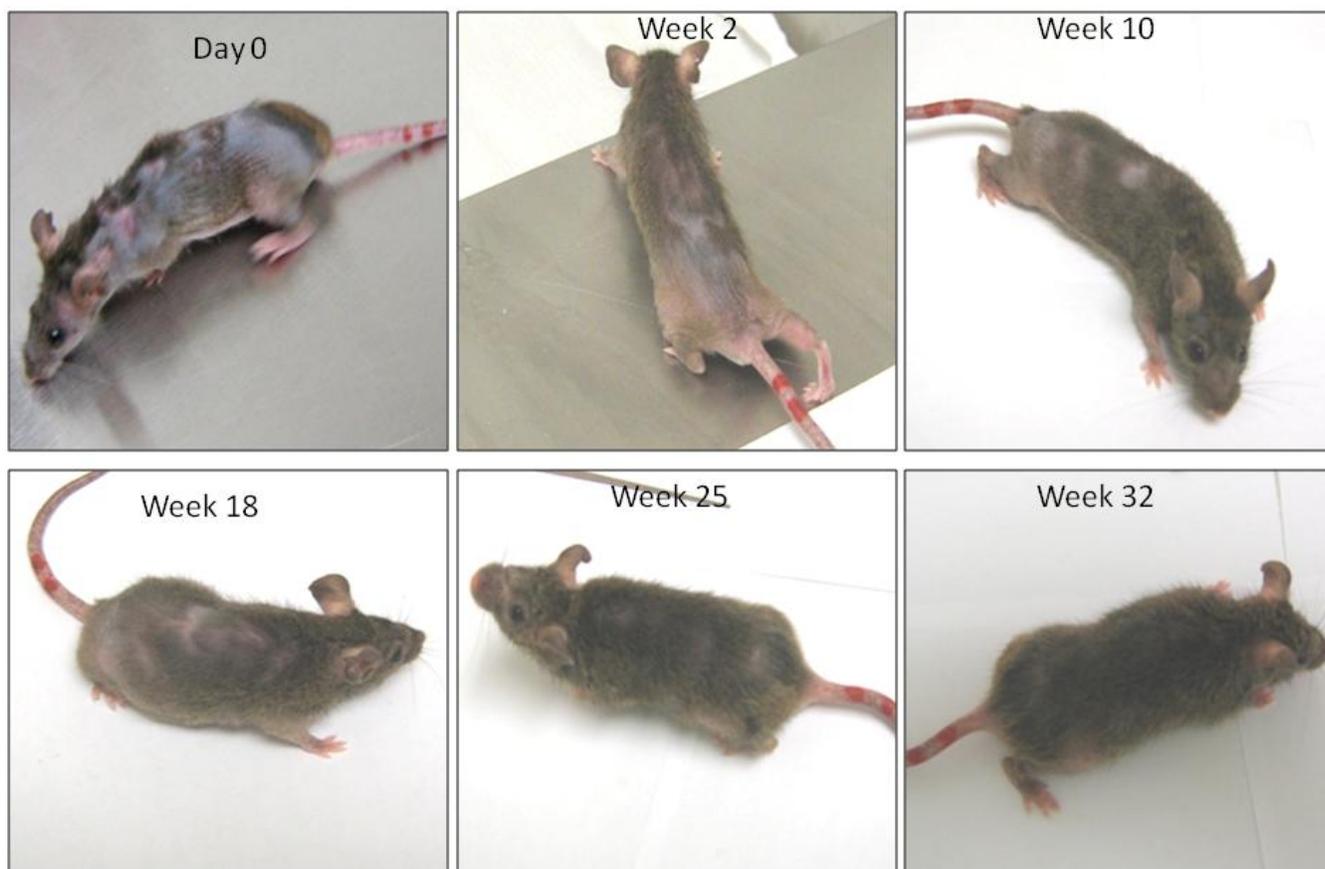
Finally, the absolute numbers of NK cells and T/B lymphocytes were obtained for the spleen and bone marrow for each mouse (CVT-E002-consuming and control), by converting these recorded percentage values via the known total cell content of each organ, the latter having been obtained from the hemocytometer at the time of organ extraction. In the case of the blood, because it was not possible to determine the entire blood volume, and therefore the total nucleated cell content of the entire blood volume for each mouse, the data were retained and presented as percentage values.

## Statistical analysis

Mean +/- standard error values are presented in the tables. The student's "t" test was performed and statistical significance between experimental and control values in each case was defined as  $p < 0.05$ .

## RESULTS

Figure 1A (upper panels: 0 - 32 weeks) shows a representative example (N=14) of the progressive, time-dependent, hair re-growth of an originally alopecic, 18



**Figure 1A:** Hair re-growth during 0 – 32 wk of CVT-E002 administration

(0-32weeks) indicate the progressive hair regrowth in a representative C3H aged 18 mo female mouse (N = 14), almost completely alopecic when dietary administration of CVT-E002 began (day 0: 80mg/day/mouse). By 2 week of daily dietary CVT-E002 (80mg/day/mouse), mice were beginning to show considerable hair re-growth and by 32 week (8 months) of consuming the extract in their chow, the hair of all mice had become fully reconstituted. Moreover, the quality of their hair was indistinguishable from that of normal (control) mice of the same age, strain and gender which had never become alopecic. At this time, mice were 26 mo (18 mo + 8 mo) of age.

months old mouse, fed daily for 32 weeks (8 months) with 80 mg CVT-E002 homogenized in the chow. Half these mice (N = 7) were euthanized at 32 week to provide the data for Table 1 A, B, C. Control mice (N = 7), not receiving dietary CVT-E002 were, at 32 weeks, physically identical to the Day 0 mouse seen in the upper 6 panels of Figure 1A. These control mice nevertheless showed no other signs of pathology.

Figure 1B (lower panels: 35 – 46 weeks) shows a representative example of a mouse from the remaining 7 mice from the original 14 (above), from which CVT-E002 had been withdrawn at 32 weeks. These 7 mice had been transferred to the control diet at 32-46 weeks. The photos in this lower panel begin at week 35, i.e., 3 weeks after being placed on the control diet, and indicate that during these subsequent 14 weeks (3 ½ months) on the control diet, these mice had retained their full body hair, and were active and healthy by all clinical indicators. The specific photo (week 46) in this panel shows a complete renewal of body hair precisely equivalent in quality/texture to that of a normal, non-alopecic mouse of this age, strain and gender.

Table 1A indicates the absolute number of NK and non-NK cells (T/B lymphocytes) in the spleens of CVT-E002-consuming and control-diet alopecic mice, while Table 1B provides the equivalent data for the bone marrow of these mice. The results indicate that in both the spleen and bone marrow, NK cells and non-NK cells were significantly increased quantitatively (absolute numbers) over their corresponding controls. Table 1C shows the relative numbers (%) of NK cells and non-NK cells (T/B lymphocytes) in the blood of the same alopecic mice. These results indicate a significant increase of NK cells and T/B lymphocytes, in relative terms (%) and reflect the significantly elevated absolute numbers of these cells in the bone marrow and spleen.

Figure 2 shows a representative mouse (N = 6), fed only 2 mg/6gm chow/day for 5 weeks. In this case, the low dose resulted in every mouse in this group demonstrating that hair re-growth only began to become evident by week 4-5. This observation is in distinct contrast to the hair re-growth dynamics of mice consuming 80mg/CVT-E002/day/mouse (Figure 1A, upper 6 panels) where mice began showing

**Table 1A.** Effect of CVT-E002 on NK cells and lymphocytes in the spleens of alopecic mice

Treatment	NK cells (x10 <sup>6</sup> ) <sup>a</sup>	Lymphocytes (x10 <sup>6</sup> ) <sup>a</sup>
None (control)	2.96 ± 0.08 <sup>b</sup> (N = 7)	36.01 ± 2.24 <sup>b</sup> (N = 7)
CVT-E002 80mg/day	10.23 ± 0.61 <sup>c</sup> (N = 7)	62.39 ± 3.03 <sup>d</sup> (N = 7)

**Table 1B.** Effect of CVT-E002 on NK cells and lymphocytes in the bone marrow of alopecic mice

Treatment	NK cells (x10 <sup>6</sup> ) <sup>a</sup>	Lymphocytes (x10 <sup>6</sup> ) <sup>a</sup>
None (control)	0.79 ± 0.08 <sup>b</sup> (N = 7)	3.42 ± 0.23 <sup>b</sup> (N = 7)
CVT-E002 80mg/day	4.98 ± 0.19 <sup>c</sup> (N = 7)	19.15 ± 1.35 <sup>d</sup> (N = 7)

**Table 1C.** Effect of CVT-E002 on NK cells and lymphocytes in the blood of alopecic mice

Treatment	NK cells (%) <sup>e</sup>	Lymphocytes (%) <sup>e</sup>
None (control)	5.2 ± 0.26 <sup>b</sup> (N = 7)	24.37 ± 0.81 <sup>b</sup> (N = 7)
CVT-E002 80mg/day	12.94 ± 1.28 <sup>c</sup> (N = 7)	32.77 ± 1.43 <sup>d</sup> (N = 7)

<sup>a</sup> Absolute numbers of cells calculated from the proportions of cells recorded (identified and enumerated) among (A) 1,000 spleen cells or (B) 2,000 bone marrow cells/smear/mouse, and the known total spleen cell numbers and bone marrow cell numbers (2 femurs) in each mouse, obtained by means of a hemocytometer at the time of animal euthanasia immediately at the conclusion of daily, dietary CVT-E002 for 32 wk. <sup>b</sup> Mean +/- s.e. <sup>c</sup> p<0.0001 vs control NK cells {(A), (B) and (C)} <sup>d</sup> p<0.0001 vs control lymphocytes {(A), (B) and (C)} <sup>e</sup> NK cells and other lymphocytes were recorded as a proportion (%) of 1,000 total cells identified and enumerated on blood smears for each mouse.

abundant hair after only 2 weeks. In other words, hair regrowth appears to be CVT-E002 dose-dependent. This low dose (2mg) of CVT-E002 was assessed in a previous dose/response study (Miller et al., 2009), to be the lowest dose where minimal increases in NK cells T/B lymphocytes were observed. In our studies (Miller et al., 2009; Durairaj et al., 2012; 2013) the dose/response relationship in adult mice consisted of progressively assessing the responses through doses of 2, 40, 80, 120mg/day/adult mouse and selecting the highest dose for which maximum stimulation of immune cell numbers in adult mice could be found. This was 80mg of CVT-E002. No further increase in the numbers of immune cells could be achieved even at 120mg CVT-E002 (Miller et al., 2009).

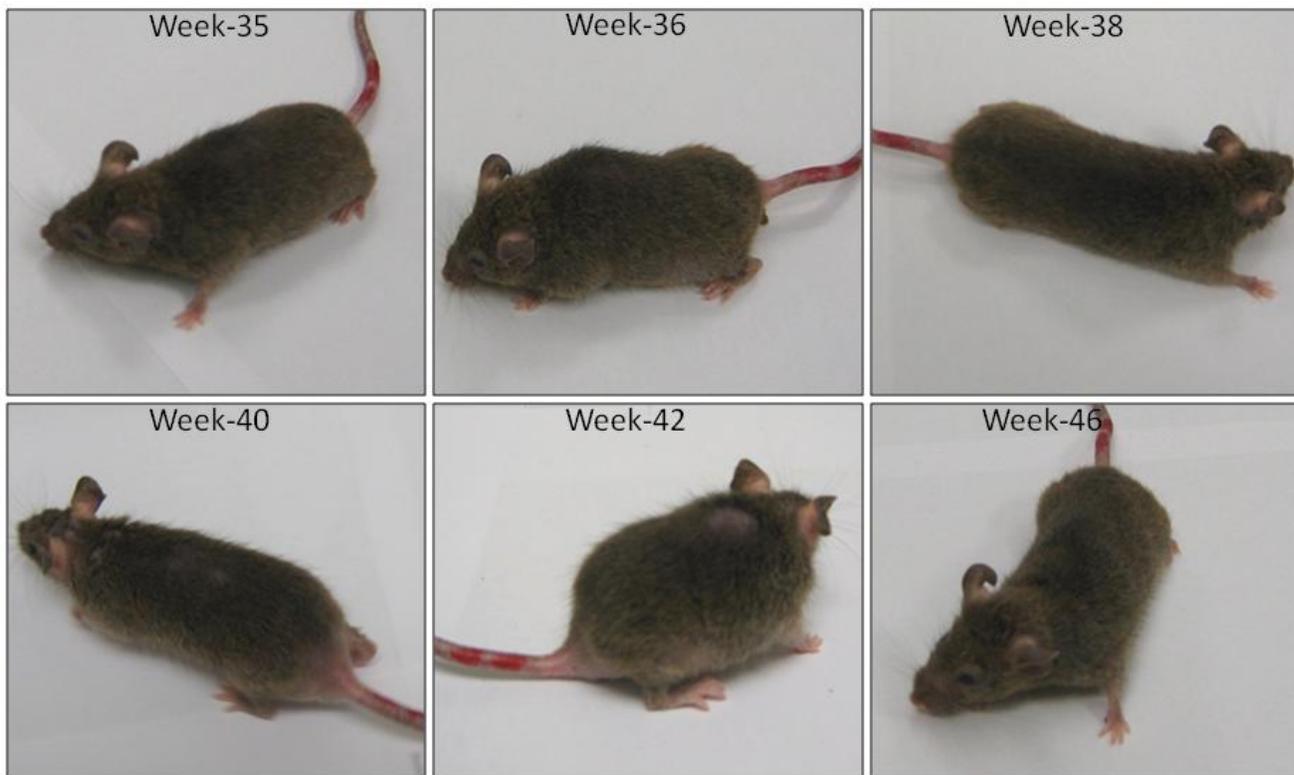
We similarly tested the dose/response relationships using 5, 10, 20, 30, and 50mg in infant and juvenile mice (Miller et al., 2011b).

Table 2 A, B, C provides the data observed when feeding 2 mg CVT-E002 and represents the same parameters assessed in Tables 1 A, B, C. The results reveal that although

2mg/day/mouse was not adequate to rapidly stimulate regrowth of the hair at the rate seen with 80mg/day/mouse (Figure 1A, upper 6 panels: 0 – 32 weeks), this low dose (2 mg) did nevertheless result in an increase in both the absolute numbers of NK cells and non-NK lymphocytes in the spleen and bone marrow. However, as evidenced by the level of statistical significance, the magnitude of stimulation for these cells in both organs, did not in every case reach the levels of significance obtained with 80mg CVT-E002 (Table 1 A, B, C). Finally, as in Table 1C, the increase in the relative numbers (%) of these cell categories in the blood versus the corresponding control-diet mice reflected the increase in the organ-based absolute numbers.

## DISCUSSION

This study has shown that CVT-E002, an extract from North American ginseng (*Panax Quinquefolius*), already in the market-place for the amelioration of respiratory afflictions



**Figure 1B:** Hair re-growth after CVT-E002 withdrawal

(35-46week) shows a representative mouse, indicating retention of whole body hair on the remaining half (N = 7) of the mice of Figure 1A upper 6 panels, after withdrawing CVT-E002 from their daily diet for the 14 wk period from 32 →46week, i.e., 3½ mo. Photos, were taken beginning 3 week after withdrawing CVT-E002, i.e., at 35 week. All 7 mice retained their body hair during these 14 wk (3½ mo). Mice were now 29½ mo (18 mo + 8 mo + 3½ mo) of age.



**Figure 2:** Hair re-growth dynamics from 0 – 5 week during low dose (2mg/day/mouse) administration of CVT-E002

The 2 photos indicate the hair re-growth status of a representative mouse (N = 6) consuming only 2mg/day/mouse of CVT-E002 daily for 5 wk (1¼ mo). In none of the 6 mice given this low dose of CVT-E002, was there any sign of the rapid, early (2 wk) hair re-growth observed with 80mg CVT-E002/day seen in Figure 1(A). Nevertheless, re-growth at this low dose was beginning, but only as fine, barely visible - but palpable - hair, and delayed until 4 -5 wk of daily low-dose (2 mg) CVT-E002 treatment. At 5 wk, all 6 mice were euthanized to provide the data for Table 2 A, B, C. At euthanasia, mice were 19¼ mo (18 mo + 1¼ mo) of age.

and well-established in humans as being free from side effects, may be a tool for ameliorating alopecia areata. While this study was not intended to be a full analytical assessment histologically, physiologically or

endocrinologically, of this complex autoimmune condition, it nevertheless, provides an innovative and justifiable basis for continued exploration of this phenomenon along these 3 avenues of research.

**Table 2A.** Effect of CVT-E002 on NK cells and lymphocytes in the spleens of alopecic mice

Treatment	NK cells (10 <sup>6</sup> ) <sup>a</sup>	Lymphocytes (10 <sup>6</sup> ) <sup>a</sup>
None (control)	3.00 ± 0.075 <sup>b</sup> (N = 6)	35.10 ± 2.14 <sup>b</sup> (N = 6)
CVT-E002 2.0mg/day	5.83 ± 0.33 <sup>c</sup> (N = 6)	44.37 ± 1.20 <sup>d</sup> (N = 6)

**Table 2B.** Effect of CVT-E002 on NK cells and lymphocytes in the bone marrow of alopecic mice

Treatment	NK cells (10 <sup>6</sup> ) <sup>a</sup>	Lymphocytes (10 <sup>6</sup> ) <sup>a</sup>
None (control)	0.77 ± 0.10 <sup>b</sup> (N = 6)	3.50 ± 0.26 <sup>b</sup> (N = 6)
CVT-E002 2.0mg/day	2.98 ± 0.23 <sup>c</sup> (N = 6)	11.96 ± 1.135 <sup>d</sup> (N = 6)

**Table 2C.** Effect of CVT-E002 on NK cells and lymphocytes in the blood of alopecic mice

Treatment	NK cells (%) <sup>e</sup>	Lymphocytes (%) <sup>e</sup>
None (control)	1.30 ± 0.097 <sup>b</sup> (N = 6)	30.93 ± 3.98 <sup>b</sup> (N = 6)
CVT-E002	2.03 ± 0.14 <sup>c</sup> (N = 6)	39.50 ± 3.74 <sup>d</sup> (N = 6)

<sup>a</sup> Absolute numbers of cells calculated from the proportions of cells recorded (defined and enumerated) among (A) 1,000 spleen cells, (B) 2,000 bone marrow cells/smear/mouse, and the known total spleen cell numbers and bone marrow cell numbers (2 femurs) in each mouse, obtained by means of a hemocytometer at the time of animal euthanasia, immediately at the conclusion of daily, dietary CVT-E002 for 5 wk. <sup>b</sup> Mean +/- s.e. (A) <sup>c</sup> p < 0.0001 vs control NK cells; <sup>d</sup> p < 0.006 vs control lymphocytes (B) <sup>c</sup> p < 0.0014 vs control NK cells; <sup>d</sup> p < 0.0001 vs control lymphocytes (C) <sup>c</sup> p < 0.0001 vs control NK cells; <sup>d</sup> NS: not significant; <sup>e</sup> NK cells and other lymphocytes were recorded as a proportion (%) of 1,000 total cells identified and enumerated on blood smears for each mouse.

NOTE the similarity in absolute numbers in the control groups in Table 2, for both cell groups, with the control groups of Table 1; By contrast, wider ranges are found in the blood data between both tables because those data represent relative numbers, indicating the interference of other cell lineages necessarily involved in computing any relative (%) values.

Two key observations have emerged from this study. First, this agent, when administered daily via the diet, to alopecic C3H mice, resulted in complete and sustained hair regrowth even after the extract was discontinued from the diet. Secondly, the daily dosage of CVT-E002 proved to be critical in achieving alopecia amelioration.

The significant increase in immune cells (NK cells and non-NK T/B lymphocytes) in all 3 organs (spleen, bone marrow and blood) in the presence of CVT-E002, *in vitro* and *in vivo*, under normal and disease conditions, is consistent with several previous observations by other laboratories as well as our own (Wang et al., 2001; 2004; Miller et al., 2009; 2011a; Durairaj and Miller, 2012; 2013). The immune privilege with which the anagen (hair follicle

growth center) is imbued under normal conditions is based on numerous carefully balanced factors. The autoimmune phenomenon itself, in any tissue so affected is complex and many events in autoimmune attacks on normal tissues remain a mystery.

In normal hair growth, immune privilege is maintained in a balance between positive and negative influences mediated by NK cells and T lymphocytes and assorted cytokines/molecular factors (McElwee et al., 2002; Gregoriou et al., 2010). A cascade of cytokines is induced from various immune cells, in the presence (*in vivo* and *in vitro*) of CVT-E002. These are IFN  $\gamma$ , IL-1, IL-2, IL-6, nitric oxide (NO) and TNF- $\alpha$  (Wang et al., 2001; 2004). Moreover, not only are cytokines pleiotropic, with actions that are

exquisitely dose and exposure time-dependent, but several cytokines can act together in a cascade manner.

Re-establishment of immune privilege at the level of the anagen would be the ultimate solution for reversing alopecia areata. At present, it is not possible to explain mechanistically the interplay between/among NK cells, other immune cells and the numerous cytokines induced by CVT-E002 (above). However, regardless of the mechanism, CVT-E002 has intervened in the autoimmune processes responsible for this condition, by tipping the balance in favor of those processes/factors/cells which stimulate hair follicle growth.

This study has shown a clearly positive influence of CVT-E002 on alopecia areata and provides a basis for studies at the molecular level together with an analysis of components interacting right within the anagen itself.

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### Conflict of Interest

The authors declare no conflict of interest with any other academic institution or commercial establishment.

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