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C-Med 100®, a hot water extract of *Uncaria tomentosa*, prolongs lymphocyte survival *in vivo*

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Summary

Water extracts of the bark of *Uncaria tomentosa,* a vine indigenous to South America, has been used for generations as an "immuno modulator". To understand the basis of this immuno modulatory effect we fed mice in their drinking water with C-Med 100®, which is a commercially available water extract from *Uncaria tomentosa*. We found a dose-dependent increase in spleen cell numbers in the supplemented mice, but the proportions of B cells, T cells, NK cells, granulocytes, and memory lymphocytes were normal. However, there were no detectable changes of the lymphoid architecture of the spleen even after long-term treatment. Further, when C-Med 100® treatment was interrupted the cellularity returned to normal level within four weeks. The increased number of lymphocytes was most likely not due to increased production because C-Med 100® did not have any significant effect on precursor cells nor on the accumulation of recent thymic emigrants in the spleen. We conclude that accumulation is most likely due to prolonged cell survival, because adoptive transfer experiments demonstrated that C-Med 100® treatment significantly prolonged lymphocyte survival in peripheral lymphoid organs, without increasing their proliferation rate. Since the accumulation was reversible and without detectable pathological effects, these results suggest the use of C-Med 100® as a potential agent for clinically accelerating the recovery of patients from leukopenia.

Key words: Cat's claw**,** cell number, lymphocyte turnover, expansion

Introduction

The vine *Uncaria tomentosa* indigenous to the Peruvian Amazon belongs to the family Rubiaceae. It is commonly known as Cat's claw or Úna de gató. Extract and decoctions of cat's claw are widely used in the Peruvian medicine for the treatment of a wide range of health problems like arthritis, inflammations, cancer, allergy and viral infections (Jones, 1995). The extracts or components of this plant have been shown to have anti-inflammatory, antiviral, antimutagenic and antioxidant activities as well as to enhance phagocytosis (Aquino et al., 1991; Aquino et al., 1989; Cerri et al., 1988; Desmarchelier et al., 1997; Keplinger, 1982; Rizzi et al., 1993; Wagner et al., 1985). Sandoval et al. have previously shown that extracts from *Uncaria tomentosa* also are remarkably potent inhibitors of TNFα-production, which suggests that a possible mechanism for the anti-inflammatory actions is immuno modulation via suppression of TNFα synthesis (Sandoval et al., 2000). In another report Sandoval-Chacon et al. (Sandoval-Chacon et al., 1998) report that extracts from *Uncaria tomentosa* negate the activation of the transcription factor NF-κB, which controls many inflammatory signals in-

Abbreviations: APC – Allophycocyanin; CFSE – arboxyfluorescein diacetate succinimidyl ester; C-Med 100® – a water extract from *Uncaria tomentosa*; Cy5 – cyanine 5; DXR – Doxorubicin; PE – Phycoerythrine; PerCP – Peridinine chlorophyll protein; WBC – white blood cells

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cluding cytokine secretion (Bäuerle and Henkel, 1994). Thus, one possible explanation for the benefical effects seen after treatment with extracts from *Uncaria tomentosa*, is that these mediate anti-inflammatory effects by direct inhibition of the activation of the transcription factor NF-κB and all the pro-inflammatory actions that are controlled by this factor.

During the past ten years, Cat's claw in different forms has been introduced in Europe to treat patients suffering from cancer and some viral diseases (Sandoval-Chacon et al., 1998). C-Med 100® is a proprietary aqueous extract from cat's claw. This product is derived through water extraction and exclusion of higher molecular weight components (>10 kD) such as tannins (Sheng et al., 2000a). Sheng et al. have shown that this extract is an effective inducer of apoptosis in HL60 leukemia cells (Sheng et al., 1998) and an enhancer of both DNA repair and immune responses *in vivo* (Lamm et al., 2001; Sheng et al., 2000a). Interestingly, both rats and human volunteers supplemented with C-Med 100® have been observed to have elevation of white blood cell (WBC) numbers (Sheng et al., 2000a). The C-Med 100® extract also stimulates the recovery of WBC in rats with Doxorubicin (DXR) induced leukopenia (Sheng et al., 2000b). In that study the Granulocyte colony-stimulating factor Neupogen® was used as a control for WBC recovery. Importantly C-Med 100® induced proportional increase in all fractions of WBC as compared to Neupogen®, which only increased the non-lymphocyte fractions of WBC. These observations suggested that C-Med 100® might influence the WBC turnover rate and/or the production of these cells in the primary lymphoid organs. Thus, this extract might potentially have important functional impact on lymphocyte homeostasis.

Lymphocyte homeostasis has during the past decade been a field of intense investigation. It is currently thought that naïve quiescent peripheral T cells are actively engaged in maintaining their own survival and that recognition of major histocompatibility complex (MHC) proteins is required for this event (Kirberg et al., 1997; Rooke et al., 1997; Takeda et al., 1996; Tanchot et al., 1997b). However, this conviction was recently questioned (Dorfman et al., 2000). CD4 or CD8 memory T cells on the other hand have been reported not to need interactions with MHC class II or MHC class I molecules for their survival (Murali-Krishna et al., 1999; Swain et al., 1999). Moreover at least among $CD8^+$ T cells, these two compartments appear to have their own autonomous homeostatic controls, that ensure the presence of naïve and memory cell pools (Tanchot et al., 1997a; Tanchot and Rocha, 1995). Taken together, the size of the total T cell pool seems highly regulated (Freitas and Rocha, 1993) and it is maintained at a remarkably constant level despite environmental antigenic stimulation and thymic output (Bell and Sparshott, 1997; Freitas and Rocha, 2000; Freitas and Rocha, 1993).

The survival of the naïve peripheral B cells also appears to involve interactions between the B cell receptor (BCR) and yet unidentified ligand(s) (Lam et al., 1997). After their production in the bone marrow, the survival of naïve B cells is associated with peripheral selection of cells that express particular VH-gene families (Freitas et al., 1990; Viale et al., 1993). These data indicate that the recognition interactions related to B cell survival may not require the involvement of the full antigen-binding site but rather it is mediated by the V_H-region.

In this paper we have studied the impact of C-Med 100® on the immune system of the mouse. Specifically, we have attempted to identify the C-Med 100® induced mechanisms involved in accelerating the recovery from leukopenia.

Material and Methods

Mice

C57BL/6 females were bought from Bomholdgård, Ry, Denmark and used in experiments at an age of 6–8 weeks. The animals were kept in a specific pathogen free (SPF) facility at Lund University.

C-Med 100®

C-Med 100® is a patented extract from Cat's claw, *Uncaria tomentosa* (U.S. patent 6,039,949), supplied by CampaMed, Inc. (New York, NY, USA). This is a water-soluble extract ultra-filtered to remove high molecular weight conjugates (>10,000 MW), containing 8–10% carboxy alkyl esters as active ingredients, and it is essentially free of oxindole alkaloids (<0.05%) (Sheng et al., 2000b). The bulk of the active ingredients of C-Med 100® (85%) absorb onto charcoal, have an UV absorption maximum of 200 nm, and react with hydroxylamine and ferric chloride, thus characterising them as esters. For treatment, the extract was dissolved in autoclaved tap water and fed to mice instead of normal drinking water. During treatment the extract was replaced every three days. The experimental groups were fed with C-Med 100® in their drinking water at approximate daily doses of 125, 250 or 500 mg/kg body weight for different periods of time.

Flow cytometry

Thymus, spleen, lymph nodes and bone marrow were dissected and single cell suspensions were prepared in HBSS. Aliquots of 10^6 cells were stained with monoclonal antibodies in FACS-buffer (HBSS supplemented with 0.1% NaN_3 and 3% FCS (Gibco BRL, Life

Technologies, Paisley, GB) as previously described (Ivars, 1992). Cells were pre-incubated for 10 min on ice with the anti-Fc receptor antibody 2.4G2 (Unkeless, 1979) (prepared in our laboratory) to prevent antibody binding to Fc-receptors. The cells were analysed with a FACSCalibur flow cytometer using the CellQuest software (Becton Dickinson, San José, CA).

Monoclonal antibodies

The following antibodies were used for flow cytometry analysis: FITC-conjugated anti-CD25 (7D4), anti-CD62L (MEL-14) (Pharmingen, San Diego, CA), anti-CD8 (YTS169.4) and anti-CD3ε (145.2C11) (prepared in our laboratory). PE-conjugated anti-NK1.1 (PK136), anti-CD45RB (16A), anti-CD4 (RM4-5), anti-CD44 (IM7), anti-CD62L (MEL-14) and anti-IgM (Pharmingen), anti-CD8 (CT-CD8α) (Caltag Laboratories, South San Francisco, CA). Cychrome-conjugated anti-CD44 (IM7), Biotin-conjugated anti-Gr-1 (RB6-8C5), Peridinin chlorophyll protein (PerCP)-conjugated anti-CD3ε (145.2C11), anti-CD8 (53-6.7), anti-CD4 (RM4-5) and Allophycocyanin (APC)-conjugated TCRβ (H57-597) (all from Pharmingen). Cyanin-5 (Cy-5)-conjugated anti-CD4 (GK1.5), anti-CD8 (YTS169.4) and anti-B220 (RA3.6B2) (prepared in our laboratory). The biotin-conjugated antibodies were revealed in a second step using Streptavidin (SA)-conjugated FITC (Southern Biotechnology Associates, INC, Birmingham, AL)

Intra thymic injection

C57BL/6 mice were anaesthetised by intra-peritoneal injection of avertin (200 µl/10 g of bodyweight). The anaesthetised mice were injected with 10 µl of 1 mg/ml FITC (isomer I, Sigma-Aldrich, Stockholm, Sweden) or 10 µl of PBS in each tymic lobe, essentially as described (Kelly and Scollay, 1990). After the injection the mice were put on a heating pad and their awakening supervised. Cells from the thymus, the auxiliary lymph nodes and the spleen were analysed by flow cytometry 24 hours after the intra thymic injection and the percentage of FITC-positive-TCRβ positive cells were determined in the different organs.

Adoptive transfer

Normal and C-Med 100® fed mice were injected intravenously with 20×10^6 pooled lymph node and spleen cells. Before transfer the cells were stained for 8 minutes at room temperature with 1 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Leiden, Holland) in PBS and thereafter extensively washed in PBS. The mice were sacrificed at various time points after transfer and spleen cells analysed by flow cytometry. The weight of the spleen and the bodyweight was also monitored in the recipients. In some experiments adoptive transfers were made to C57BL/6 mice that had been irradiated (300 Rad) 24 hours prior to cell transfer.

Cell cultures

Spleen cells were prepared and cultured in RPMI medium (Gibco BRL) supplemented with 10% fetal calf serum, 10 mM HEPES buffer, antibiotics, 50 µM 2-mercaptoetanol and 1 mM sodium pyruvate (all supplements from Gibco) at 37 °C, 5% CO_2. The cultures were made in 24-well plates (Costar, Cambridge, MA). To study cell survival the number of live and dead cells were determined in triplicate cultures by trypan blue exclusion. CFSE stained cells (see above) were cultured in the same way and polyclonally stimulated either by 1 µg/ml anti-CD3 antibody (145.2C.11) or by 10 µg/ml lipopolysaccharide (LPS) (Difco, Detroit, MI, USA). Proliferation was assayed by including 1 µCi of ^{3}H-Thymidine during the last four hours of culture.

Immunohistochemistry

Spleens were fixed in pentane and thereafter cut in 6–7 µm thick sections using a JUNG CM3000 Cryostat (Leica, Stockholm, Sweden). The sections were first pre-incubated with (10mg/ml) anti-Fc receptor 2.4G2 antibody. Sections were thereafter stained with biotin-conjugated anti-B220 (RA3.6B2) antibody followed in a second step by Extravidin alkaline phosphatase conjugate and revealed in a third step using Fast Fast Red TR/NAPHTHOL AS MX*TA (Sigma-Aldrich, Stockholm, Sweden AB). After this, the sections were preincubated with 10% goat serum (Dakopatts AB, Älvsjö, Sweden) and 20% fetal calf serum (Gibco) in PBS. Anti-TCRβ (H57) was then added followed in a second step with biotin-SP-conjugated affiniPure goat anti-Armenian hamster IgG (H+L) antibody (Jackson ImmunoResearch Laboratories INC, West Grove, PA). The sections were thereafter incubated with peroxidase Vectastain Elite ABC Kit (Immunkemi F&D AB, Järfälla, Sweden) and finally the labelled cells were visualised with the peroxidase substrate kit – Vector SG (Immunkemi F&D AB).

Statistics

Comparison of mean differences between two groups was made using Student's two-tailed t-test for unequal variance.

Results

Increased spleen cell number in C-Med 100® treated mice

To study the impact of C-Med 100® on the immune system, mice were fed with various concentrations of

C-Med 100® in the drinking water and cells from lymphoid organs were analysed after various periods of treatment. The number of spleen cells had increased in mice treated with C-Med 100® (Fig. 1A) as compared to controls receiving non-substituted drinking water. The increase was dose-dependent (Fig. 1A) and had reached plateau levels at about 3 weeks of treatment (data not shown). In contrast, however, the total number of thymocytes did not change (data not shown) suggesting that the treatment influenced numbers of peripheral cells only. The number of cells in the lymph nodes and bone marrow could not be appropriately compared because of highly variable recovery of cells from individual mice within the various experimental groups. Neither the body weight nor the spleen weight changed significantly by the treatment (Fig. 1B). Importantly, consumption of drinking water was not reduced even when substituted with the highest concentration of C-Med 100® (4 mg/ml; corresponding to about 500 mg/kg body weight/day).

To determine what cells had increased in number by C-Med 100® treatment, we analysed spleen and lymph node cells by flow cytometry. We found that the proportion of lymphocyte subsets, NK cells and granulocytes were normal, indicating that the treatment had not significantly changed the cellular composition (Table 1). Consequently, the absolute numbers of splenic T cells ($p = 0.02$), B cells ($p = 0.03$), NK cells ($p = 0.009$) and NKT cells ($p = 0.02$) had significantly increased (Fig. 1C) in the treated mice ($n = 7$). However, the increase in absolute cell number of granulocytes (Gr-1$^+$) was not statistically significant ($p = 0.07$), probably due to the high variability between individual animals.

To determine whether the increased number of lymphocytes would be due to the accumulation of either naïve or "memory" cells we analysed the expression of various membrane markers by lymphnode CD4$^+$ T cells. As can be seen, the proportion of naïve CD4$^+$ CD62L^{hi}, CD45RB^{hi} and memory/activated CD4$^+$ CD44^{hi} CD45RB^{low} CD69$^+$ cells were identical in normal and treated mice (Fig. 1D). Thus, the in-

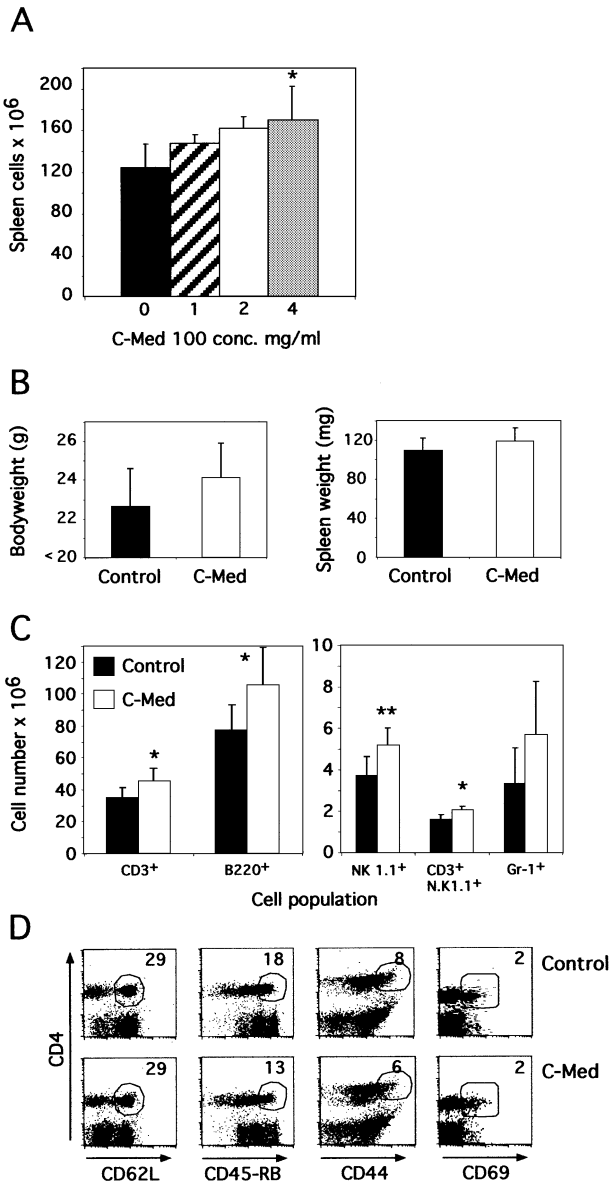

Fig. 1. (A) Accumulation of leukocytes after 24 days of C-Med 100® treatment. Groups of mice were treated with indicated concentrations of C-Med 100® in the drinking water. The absolute spleen cell numbers in individual mice were determined by trypan blue exclusion. The presented data for the control group and for the 4.0 mg/ml C-Med 100® group are from two experiments involving a total of seven mice per group. The presented data for the 1.0 mg/ml and 2.0 mg/ml C-Med 100® groups are from one experiment involving a total of two mice per group. The data are presented as mean cell number ±SD. (*) Statistically significant difference ($p < 0.05$) compared to the control group. (B) The body weight and spleen weight from mice treated with 4 mg/ml C-Med 100® for 25 days or from untreated controls. The presented data are pooled from two experiments involving a total of seven mice per group. The data are presented as means ±SD. (C) Spleen cells from mice treated with 4 mg/ml C-Med 100® for 25 days or untreated controls were stained by antibodies to indicated cell surface markers and analyzed by flow cytometry. The figure shows the absolute number of cells with the indicated phenotype and represents means of seven mice/group ±SD. (*) (**) Statistically significant differences ($p < 0.05$) and ($p < 0.01$), respectively, compared to the control group. (D) Lymph node cells from mice treated with 4.0 mg/ml of C-Med 100® for 25 days or from untreated controls were stained with antibodies to indicated cell surface markers and analyzed by flow cytometry. The data from one representative mouse out of five in one experiment are shown. The numbers indicate the percentage of CD4$^+$ T cells with indicated phenotype.

creased number of T cells in the C-Med 100® treated mice did not depend on increased recruitment of cells into the memory T cell pool, nor on a higher number of activated CD69+ cells. We also analyzed the expression of costimulatory molecules on dendritic cells (CD11c+) from these animals (Table 1). Again, the cells from treated mice were phenotypically identical to cells from normal mice. Taken together, our data suggest that C-Med 100® treatment results in accumulation of leukocytes without the selection for particular subsets.

Long term treatment with C-Med 100®

To study the long-term effects of C-Med 100® treatment on cell numbers, mice were treated for 63 days. The number of spleen cells was significantly increased in long term treated mice as compared to untreated controls (Fig. 2A), in accordance with the above data (Fig. 1A). Since the proportion of T and B cells remained normal, there was a significant increase in the absolute number of these subsets (data not shown). The lymphoid architecture of the spleen appeared normal with distinct T and B cell areas (Fig. 2B, left panel).

To assess whether the increase in spleen cell number would be dependent on continuos exposure to C-Med 100®, the treatment was interrupted and the animals were analysed at various time points after withdrawal. As shown in Fig. 2A the number of spleen cells returned to normal levels within four weeks. The proportion of various leukocyte populations was not changed after the withdrawal (data not shown) and the lymphoid architecture of the spleen was similar to that of untreated controls (Fig. 2B, right panel).

Taken together, these results indicated that the C-Med 100® extract contained components that increase the number of leukocytes in peripheral lymphoid organs without detectable influence on lymphoid architecture. Further, this phenomenon was reversible as the number of cells returned to control level after withdrawal of C-Med 100®, without detectable changes in cellular composition in peripheral lymphoid organs. There are at least two possible causes for the observed increase in cell numbers. Either it could be due to increased cellular development/production in the primary lymphoid organs or increased survival of peripheral leukocytes.

The impact of C-Med 100® on cell maturation in primary lymphoid organs

To test whether C-Med 100® would influence the maturation of lymphocytes, we determined the composition of precursor cell populations in the primary lymphoid organs by flow cytometry. To this end, mice were treated with C-Med 100® for 25 days and bone marrow cells and thymocytes from these mice were stained with various antibodies to reveal B and T cell precursors, respectively. The results showed that the proportion of bone marrow cells defined by B220 and IgM markers (Fig. 3A) were normal after 25 days of C-Med 100® treatment. Further, the proportions of triple negative thymocyte populations (surface negative for CD4, CD8 and CD3ε) defined by the expression of CD44 and CD25 molecules (Fig. 3B) and of

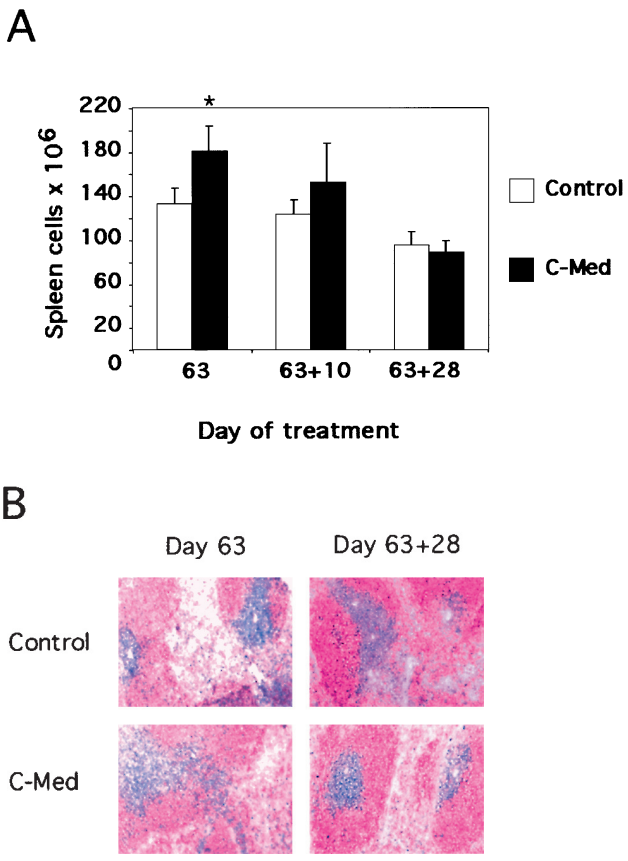

Fig. 2. The impact of C-Med 100® on leukocyte number is reversible. (A) Mice were treated with 4 mg/ml of C-Med 100® in the drinking water for 63 days. The treatment was terminated and absolute numbers of spleen cells determined, either immediately or after 10 or 28 days as indicated and compared to spleen cells from normal untreated controls. The data represent the mean ± SD of four mice per group from one representative experiment. (*) Statistically significant difference ($p < 0.05$) compared to the control group. (B) Sections of spleens from controls, from mice treated with C-Med 100® for 63 days and from mice untreated for 28 days after terminating the 63 day-treatment were stained with B220 and TcRβ antibodies in red and blue respectively (see Materials and Methods). Representative areas are shown and demonstrate the similarity of lymphoid architecture in normal untreated and C-Med 100® treated animals.

the four major thymocyte subsets, defined by CD4 and CD8 expression (data not shown), were both normal in the treated mice.

We also investigated the possibility that C-Med 100® would increase the output rate of mature T cells from thymus. C-Med 100® treated mice were given intra-thymic injections with FITC or PBS after 24 days of treatment. The spleen and the thymus of injected animals were analysed for FITC-positive cells 24 hours after the injections. The fraction of FITC-labelled thymocytes was similar in treated and untreated mice, indicating that they had been labeled with equal efficiency. Most importantly, C-Med 100® treated mice and control mice had a similar fraction of FITC-positive TCRβ-positive cells in the spleens after the FITC-injections (Fig. 3C). Taken together, these results indicated that the C-Med 100® extract did not significantly affect precursor cell maturation in primary lymphoid organs. Thus, increased production of lymphocytes was most likely not a major cause for the clear-cut increase in lymphocyte numbers observed in the treated mice.

The impact of C-Med 100® on cell survival *in vivo*

To address the possibility that C-Med 100® would influence the peripheral survival of lymphocytes, we performed adoptive transfer experiments. Spleen cells from C-Med 100® treated mice and control mice were stained with CFSE and transferred to either normal or C-Med 100® treated recipients. To estimate the lymphocyte turnover rate the decay of the CFSE positive cells was studied in the recipients. Thus, the total number of CFSE positive cells was quantified in spleens of recipient mice at various time points after cell transfer (Fig. 4A). One group of recipients was analysed three days after transfer to control for differential take of the transferred cells. We observed a slightly variable number of transferred cells in the spleens of the recipients in the various experimental groups. The difference between untreated control and C-Med 100® treated recipients respectively might be due to an increased competition for survival in the C-Med 100® treated hosts (Begon et al., 1990; Pianka, 1976). Thus, the higher number of cells already present in the C-Med 100® treated hosts could have increased the competition for essential resources (Tilman, 1982) and more of the transferred cells might have died during the first few

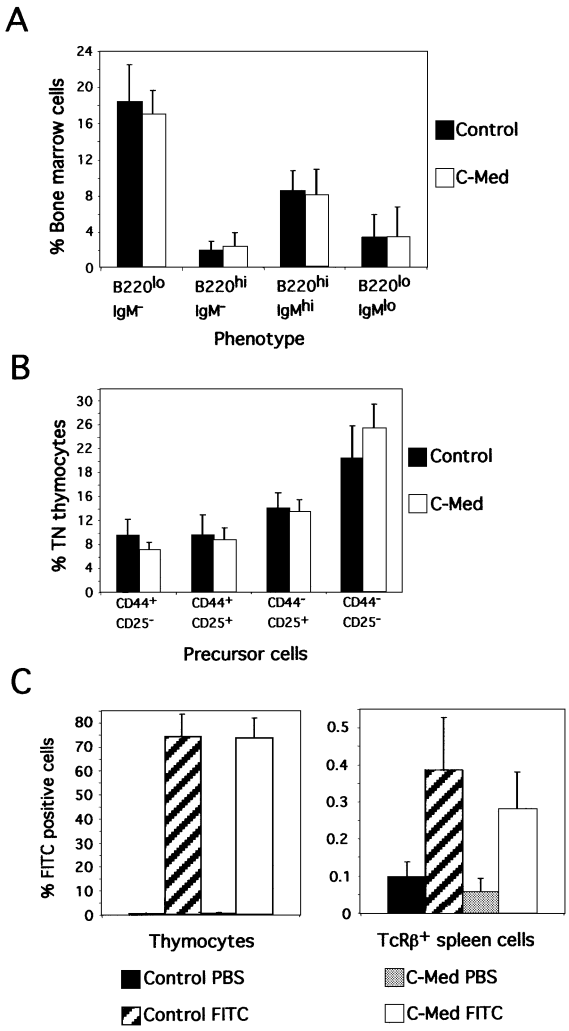

Fig. 3. The cellular composition of primary lymphoid organs is unchanged by C-Med 100® treatment. (A) Bone marrow cells from mice treated with 4 mg/ml of C-Med 100® for 25 days or from normal controls were stained with antibodies to the indicated surface markers and the cells analysed by flow cytometry. The percentage of various populations defined by these markers out of total bone marrow cells are indicated. The data represent the mean of a total of seven mice from two independent experiments with similar results. (B) Thymocytes from the same mice were stained with a cocktail of CD3, CD4 and CD8 antibodies and triple negative (TN; $CD3^-CD4^-CD8^-$) precursor cells gated in the flow cytometer. The fraction of the four sub-populations defined by expression of the CD25 and CD44 markers out of total TN cells was then determined. The data represent the mean percentage ±SD of a total of five mice per group from one representative experiment. (C) C-Med 100® treatment does not influence thymic output of T cells. C57BL/6 mice treated with 4mg/ml of C-Med 100® for 24 days or untreated controls were injected either with PBS (10 µl/lobe) or FITC (1 µg/ml, 10 µl/lobe). Thymocytes and spleen cells were prepared 24 hours later and the fraction of FITC-positive thymocytes (left panel) and $TcR\beta^+$ FITC-positive spleen cells (right panel) determined by flow cytometry. The data represent the mean percentage out of total cells ±SD of four mice per group and are representative of two independent experiments.

days after transfer. With time, the number of CFSE positive cells decayed (Fig. 4B), which enabled us to estimate the decay rate of the transferred cells.

In order to more accurately estimate the decay we first assured that the transferred cells were not dividing in the hosts, as this would lead to underestimation of the true decay rate. Since dividing cells loose CFSE-staining intensity, non-dividing cells can be positively identified (Fig. 4C). As the fraction of cells that had divided between day 3 and day 21 was very similar in the various groups (Table 2), we calculated the decay rates between these two time points using the least square method. The calculated decay rates of untreated control splenocytes and C-Med 100® treated splenocytes in control hosts, was 0.98 and 0.43 respectively. These data indicated that C-Med 100® treated splenocytes have an advantage in *in vivo* survival compared to splenocytes from untreated mice. When the same cell suspensions were transferred to C-Med 100® treated recipients, we observed decay rates of 0.69 and 0.60, respectively. The slower decay rate of the normal cells observed in this case, might be due to that the cellular lifespan is prolonged due to the presence of C-Med 100®. However, as argued above there are more cells in these hosts, which might lead to competition and a slightly higher decay rate of C-Med 100® treated cells than in the normal host. Very similar decay rates were observed from day 3 to day 36, but the fraction of cells that have divided in the various recipients is more variable leading to a less precise estimate of true cell decay.

These results are in accordance with the hypothesis that C-Med 100® may decrease the decay of lymphocytes *in vivo*. Further, the observation that NK-cells also significantly accumulated in the C-Med 100® treated mice (Fig. 1C, Table 1) suggests that the lifespan of other leukocytes might be prolonged as well.

In control experiments we transferred cells from treated mice into irradiated normal or C-Med 100® treated recipients. Transferred lymphocytes expand in such lymphopenic mice (Bell et al., 1987; Rocha et al., 1989). We observed that a slightly larger fraction of the spleen cells from C-Med 100® treated mice compared to normal control spleen cells had not divided in the untreated lymphopenic hosts 21 days after transfer (data not shown). Importantly, this difference in cell proliferation was not observed when C-Med 100® treated spleen cells were transferred to normal non-irradiated hosts or to non-irradiated C-Med 100® treated hosts (Table 2).

The impact of C-Med 100® extract on cell division *in vitro*

To determine whether *in vivo* C-Med 100® treated lymphocytes would respond normally to activation signals, we performed *in vitro* proliferation experiments. Spleen cells from normal untreated mice and from mice treated with C-Med 100® for 24 days were stimulated with T and B cell mitogens *in vitro*. The prolifera-

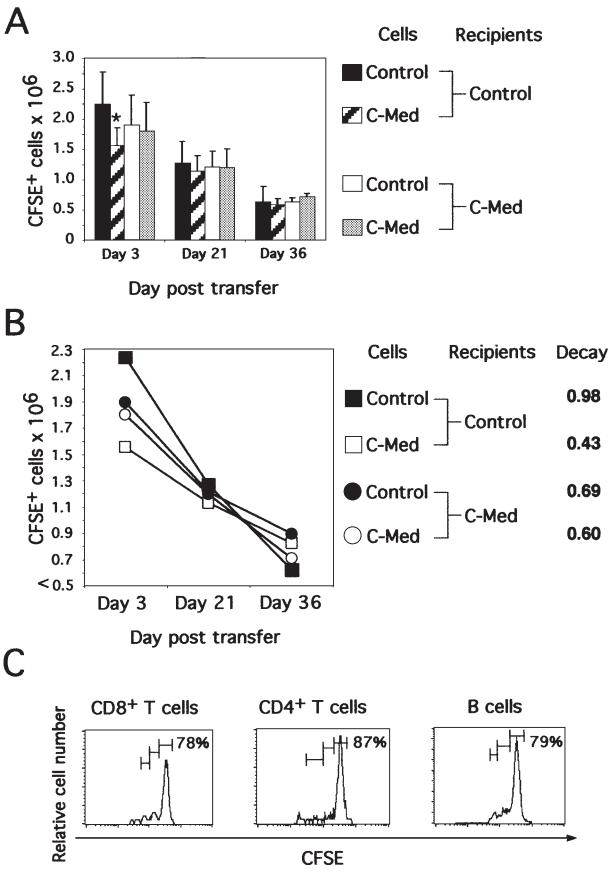

Fig. 4. Retarded decay of adoptively transferred C-Med 100® treated lymphocytes. (A) Spleen cells either from normal controls or from mice treated with C-Med 100® (4 mg/ml in the drinking water) for 25 days were stained with CFSE and transferred intravenously (20×10^6 per mouse) to normal or C-Med 100® treated recipients (4 mg/ml for 25 days) as indicated. The latter recipients were treated with 4 mg/ml C-Med 100® throughout the experiment. The total number of CFSE-positive spleen cells in the recipients was determined by flow cytometry 3, 21 and 36 days after the transfer. The results represent the mean absolute number of CFSE-positive cells ±SD of four mice per group and are representative of two experiments with similar results. (*) Statistically significant difference ($p < 0.05$) compared to the control group. (B) The means of absolute numbers of recovered CFSE-positive cells in (A) are shown. The decay rates for the different test groups were calculated between day 3 and day 21 using the least square method, and were as indicated. (C) CFSE fluorescence intensity in indicated lymphocyte populations from a C-Med 100® treated donor (see B) was determined by flow cytometry 21 days after transfer to a normal recipient mouse. The percentage of non-dividing cells is indicated. The data from one representative recipient out of eight are shown.

tion response of cells from C-Med 100® treated mice when stimulated with anti-CD3 antibodies (Fig. 5A) or LPS (Fig. 5B) was equal to that of control cells. Further, using CFSE-labeled cells we determined that the fraction of dividing cells in such cultures were very similar (Table 3). These data taken together indicate that C-Med 100® treated and normal lymphocytes proliferated equally efficiently *in vitro* and *in vivo* as shown above (Table 2).

We also studied the survival of C-Med 100® treated cells in unstimulated *in vitro* cultures (data not shown). We found that the C-Med 100® treated cells decayed at the same rate as cells from control mice. This result suggested that the cell autonomous prolonged life span of C-Med 100® treated lymphocytes observed *in vivo* (Fig. 4B) might be dependent on additional parameters not available in the *in vitro* culture system.

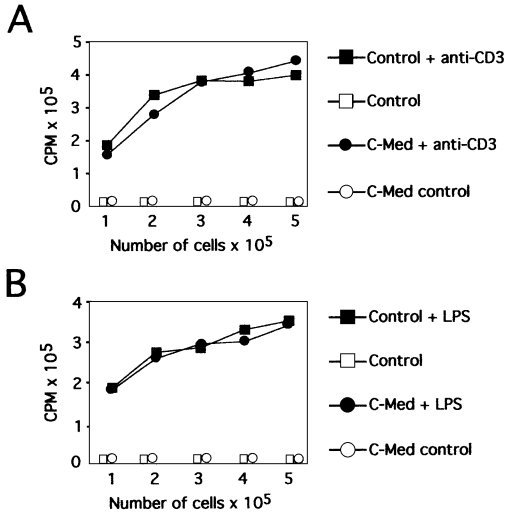

Fig. 5. Equal *in vitro* proliferation of C-Med 100® treated and normal lymphocytes. Indicated number of spleen cells from C-Med 100® treated (24 days, 4 mg/ml C-Med 100®) and normal mice were activated with A) anti-CD3 antibodies (1 µg/ml) or B) LPS (10 µg/ml). Proliferation was assayed by ^{3}H-Thymidine incorporation at day 3 of culture. One representative experiment out of two (A) and three (B) experiments performed are shown.

Discussion

A previous report demonstrated the accelerated recovery of cell-numbers in C-Med 100® treated rats with Doxorubicin-induced leukopenia (Sheng et al., 2000b). In the present study, we observed a dose-dependent increase in spleen cell number in C-Med 100® treated mice. There was no detectable change in the proportions of the various leukocyte populations studied. Further, the proportion of memory T cells in the treated mice was also unchanged, indicating that treatment with C-Med 100® extract did not induce the accumulation of certain leukocyte subpopulations only.

Long term treatment with C-Med 100®, despite the considerable increase in cell number, did not detectably change the lymphoid architecture of the spleen. Most importantly, withdrawal of the treatment led to decreased cell numbers and normalisation within 28 days of withdrawal. Again immunohistochemistry studies of the spleen of such mice revealed normal lymphoid architecture. We did not find any significant change in

Table 1. Normal proportion of leukocyte populations and expression level of co-stimulatory molecules by DCs in the spleen of C-Med 100® treated mice. The data show mean percent positive cells ± SD and the mean of Geometric mean fluorescence intensity ± SD of indicated markers from 7 and 10 mice respectively in two independent experiments. There were no statistically significant differences ($p > 0.05$) between cells from treated and untreated mice.

Cells	% positive cells				
	CD3	B220	NK1.1	CD3/NK1.1	Gr-1
Spleen					
Control	28 ± 5.4	62 ± 4.4	3 ± 0.4	1.4 ± 0.1	2.8 ± 1.4
C-Med	27 ± 3.1	61 ± 3.5	3 ± 0.7	1.3 ± 0.2	3.5 ± 1.7
	Geometric mean fluorescence				
	MHC II	CD40	CD80	CD86	
CD11c$^+$ spleen					
Control	136 ± 22.5	27 ± 8.3	40 ± 18.12	67 ± 13.1	
C-Med	140 ± 16.8	26 ± 7.5	37 ± 15.8	59 ± 8.3	

the composition of precursor B cells in the bone marrow and precursor T cells in the thymus. Further, the output of mature T cells from the thymus was normal. Even though we did not study the output of B cells and other leukocytes from the bone marrow, we tentatively concluded that C-Med 100® treatment mainly influence peripheral leukocytes. All together, these observations suggest that the C-Med 100® extract affects a basic mechanism(s) influencing leukocyte survival in peripheral lymphoid organs.

The use of adoptive cell transfers allowed us to directly test the possibility that the accumulation of lymphocytes in the treated mice would be due to increased cellular half-life. Indeed the transfer experiments supported this view as we found that lymphocytes from C-Med 100® treated mice decayed more slowly than lymphocytes from normal untreated mice when transferred to normal recipients. This observation provided an explanation to the increased spleen cell numbers, as decreased decay rate would lead to the accumulation of leukocytes in the peripheral lymphoid organs. Furthermore, these data indicated that the treatment induced a cell autonomous change that prolonged the *in vivo* survival of the cells. In parallel control experiments, the same cell populations were transferred to C-Med 100® treated recipients. We noted that C-Med 100® treated lymphocytes decayed faster in the C-Med 100® treated hosts than in untreated hosts. This phenomenon might be related to the increased number of cells in lymphoid organs of these hosts, which might increase the competition for survival since the access of space and nutrients (Freitas et al., 1996; Freitas et al., 1995) would be more limited. The slower decay of normal lymphocytes in the treated host might be due to the observed beneficial effect of C-Med 100® on the decay, but the higher cellularity of that recipient might again counteract the beneficial effect.

Sheng et al. (Sheng et al., 2000a) have previously shown that C-Med 100® extract enhances DNA repair of both single strand breaks and double strand breaks that have been induced by radiation *in vivo*. In another study Sheng et al. (Sheng et al., 2000a) showed that DNA-repair of single strand breaks were enhanced by C-Med 100® extract also when DNA damage was induced by Doxorubicin (DXR). Sandoval-Chacón et al. (Sandoval-Chacon et al., 1998) have reported that extracts of *Uncaria tomentosa* both protect cells against oxidative stress and prevent the activation of NF-κB. These effects may both prolong cellular life spans, because they would be expected to counteract induction of apoptosis, first by protection from oxidative stress and second by enhancing DNA repair. Sheng et al. (Sheng et al., 1998) have reported that C-Med 100® in-

Table 2. The fraction of non-dividing adoptively transferred lymphocytes is similar in C-Med 100® treated and normal recipients. Spleen cells from the mice in Fig. 4 were stained with antibodies to the CD4, CD8 and B220 cell surface markers. The fraction of non-dividing CFSE-stained cells in indicated lymphocyte populations, was determined with flow cytometry. The numbers are mean values of eight mice from two independent experiments.

Donor:	Control	C-Med	Control	C-Med
Recipient:	Control		C-Med	
Day	% non-dividing $CD4^+$ cells			
3	87	87	86	83
21	79	80	77	75
36	64	69	68	68
Day	% non-dividing $CD8^+$ cells			
3	88	91	90	87
21	75	75	72	72
36	62	60	59	54
Day	% non-dividing $B220^+$ cells			
3	85	84	81	83
21	81	83	80	83
36	77	77	75	77

Table 3. Mitogen-induced proliferation of lymphocytes from normal control and C-Med 100® treated mice. CFSE-labeled spleen cells from normal mice or mice treated for 25 days with C-Med 100®, were cultured *in vitro* (5×10^5 cells/ml) in the presence of ConA (2.5 µg/ml) or LPS (10 µg/ml). The cultures were harvested every 24 hrs and analysed by flow cytometry, by gating for CFSE-stained cells with indicated phenotype.

Cell culture				
Cell	Mitogen	Day 2	Day 3	Day 4
		% non-dividing $CD4^+$ cells		
Control	ConA	33	79	88
C-Med	ConA	10	64	82
		% non-dividing $CD8^+$ cells		
Control	ConA	48	86	91
C-Med	ConA	21	79	92
		% non-dividing $B220^+$ cells		
Control	LPS	24	85	90
C-Med	LPS	16	84	87

duce apoptosis in HL-60 leukemic cells while K562 cells were more resistant to this effect. We show in here that lymphocytes from C-Med 100® treated mice proliferated normally after *in vitro* stimulation. We have subsequently shown that similar concentrations of C-Med 100® as the ones used before (Sheng et al., 1998) do not induce detectable apoptosis in murine lymphocytes *in vitro*. In addition C-Med 100® did not induce apoptosis in the 70Z/3 and Raji cell lines either (C. Åkesson, unpublished data). Thus, it appears that C-Med 100® induce apoptosis in certain cell lines only.

We did not detect any significant differences in *in vitro* survival of spleen cells from C-Med 100® treated and normal mice in the absence of C-Med 100® addition to the cultures (data not shown). At present we do not know whether this finding only reflects differences between the *in vivo* and *in vitro* conditions or whether it indeed shows that the cells from treated mice are not inherently less sensitive to "spontaneous" cell death. Future experiments in this system will address the role of pro- and anti-apoptotic genes in the longevity of the treated lymhocytes.

We thought it was important to confirm that the cells from treated mice would be functionally competent. First, we demonstrate that mitogenic activation *in vitro* induced identical level of T and B cell proliferation in cells from treated and from control mice. Second, an equal fraction of the cells responded to the T and B cell mitogens in these cultures. Thus, the cells that had accumulated in the C-Med 100® treated mice appeared functionally normal. In the adoptive transfer experiments we saw that an equal fraction of treated and non-treated cells initiated proliferation in the recipients. These data indicated that also the *in vivo* proliferation of these cells was normal. The reported beneficial effect of C-Med 100® treatment on antibody production to bacterial polysacharides in human (Lamm et al., 2001), suggests that the cells accumulating after treatment would respond to antigen-specific activation *in vivo* as well.

How are our present findings related to the previously described anti-inflammatory effects of extracts from *Uncaria tomentosa*? The C-Med 100® extract is complex and may contain many different active components. Therefore, it is possible that the accumulation of leukocytes is not directly related to those effects. Rather the anti-inflammatory effects might be due to the inhibition of TNFα-production, previously reported by another laboratory (Sandoval et al., 2000). The accumulation of lymphocytes, on the other hand, might be due to the anti-oxidative effects and the effects on DNA-repair. The identification of the active components of the C-Med 100® extract should enable us to more precisely dissect and distinguish the various effects of the extract on treated animals.

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