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Spatial distribution, kinetics, signaling and cytokine production during homeostasis driven proliferation of CD4+ T cells

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Abstract

During recovery from lymphopenia, the naïve T-cells undergo homeostasis driven proliferation (HDP) and acquire a memory phenotype. The HDP of T-cells requires signals derived from T-cell-receptor, p56lck kinase, IL-7R and IL-15R. However, the role of other signaling molecules during HDP of CD4+ T-cells remains speculative. The differentiation of naïve T-cells into Th1/Th2/Th17 or Treg populations during HDP is not well understood. Present report describes the spatial and signaling characteristics of HDP of CD4+ T-cells and their cytokine profiles. The HDP of CD4+ T-cells was found to occur only in specific areas (T-cell zones) of secondary lymphoid organs of lymphopenic mice. The inhibitors of MEK and PKC and their combination with inhibitors of PI3kinase and mTOR suppressed mitogen induced T-cell proliferation without affecting their HDP. The CD4+ T-cells taken from reconstituted lymphopenic mice showed activation of proteins involved in NF-κB pathway, significantly higher production of pro-inflammatory cytokine IL-6, and lower production of IL-4 as compared to T-cells from normal mice. Plumbagin, a known NF-κB blocker inhibited survival as well as HDP of CD4+ T-cells and IL-6 production in activated T-cells. Our results demonstrate the essential role of NF-kappaB during HDP of T-cells.

Keywords

NF-κB; IL-6; Plumbagin; Lymphopenia; Inflammation

1. Introduction

T cells undergo homeostasis driven proliferation (HDP) under lymphopenic conditions suggesting that they can sense the 'empty lymphoid space' surrounding them, but the mechanisms that regulate this proliferation are not fully understood (Rocha et al., 1983). Interactions of T cell receptor (TCR) with self major histocompatibility complex (MHC) are required for prolonged survival of T cells (Kirberg et al., 1997; Tanchot et al., 1997). The same ligand receptor interactions have been shown to induce HDP of T lymphocytes during lymphopenic conditions (Khaled and Durum, 2002; Moses et al., 2003). It has also been surmised that HDP of T cells can be initiated by low affinity self peptides similar to those involved in positive selection of T cells in thymus (Ernst et al., 1999; Goldrath and Bevan,

1999; Surh and Sprent, 2000). Such an expansion could, in theory, promote selective proliferation of potentially self reactive clones leading to autoimmunity (Theofilopoulos et al., 2001).

Little is known about the TCR signaling events that result in proliferation during lymphopenia. Studies with lck and fyn knockout mice have suggested that HDP of T cells may be driven by weak or partial agonist signals that depend on lck activity (Rocha et al., 1983; Seddon et al., 2000). Recently, we showed a non-obligatory role of mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3kinase) during HDP of CD4+ T cells in vivo. Chlorophyllin, a phytochemical antioxidant, inhibited HDP as well as mitogen driven proliferation (MDP) of CD4+ T cells without inducing cell death (Sharma et al., 2007a,b). However, the role of NF- κ B, protein kinase C (PKC) and mitogen-activated kinase kinase (MAPKK or MEK) during HDP of CD4+ T cells and the differentiation of naïve CD4+ T cells into different effector populations (Th1, Th2, Th17, T follicular helper and Treg) during HDP remain to be investigated. This knowledge will facilitate the search for modulators of signaling interactions and for prevention of autoimmune diseases following HDP (Baccala and Theofilopoulos, 2005).

In the present studies we examined the role of NF- κ B, MEK and PKC during survival and HDP of CD4+ T cells in lymphopenic mice. The cytokine profile of T cells recovered from reconstituted lymphopenic mice was also investigated.

2. Materials and methods

2.1. Reagents and chemicals

The following chemicals were obtained from Sigma Chemical Company, USA: Ethylenediamine-tetra-acetic acid (EDTA), 2-mercaptoethanol (2-ME), phenylmethanesulfonyl fluoride (PMSF), igepal (NP40), trizma base, propidium iodide (PI), sodium azide, tritonX-100, tween 20, HEPES, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA), sodium chloride, sodium dihydro-phosphate, disodium-hydro-phosphate, plumbagin, RPMI 1640 medium, sodium bicarbonate, PD98059 (a PKC inhibitor), penicillin and streptomycin. Fetal bovine serum (FBS) was obtained from GIBCO BRL. Rapamycin (an mTOR inhibitor), Ly294002 (a PI3kinase inhibitor), Gö6983 (a MEK inhibitor), and concanavalin A (Con A) were purchased from Calbiochem, USA. Carboxy fluorescein diacetate succinimidyl ester (CFSE) was procured from Molecular Probes, The Netherlands. Following primary antibodies for western blotting were purchased from Cell Signaling Technologies (USA): antibodies to mouse phospho-atypical kinase (pAKT), phospho-c-Jun N-terminal kinase (pJNK), actin- β and inhibitor of kappaB- α (I κ B- α). Horse radish peroxidase (HRP) conjugated secondary antibody and western blotting kits were obtained from Roche (Germany). The phycoerythrin (PE) conjugated anti-mouse CD3 and isotype control were obtained from BD Bioscience (USA). ELISA sets for detection of different cytokines were obtained from BD Bioscience (USA). Magnetic nanoparticle conjugated antibodies for enrichment and purification of CD4+ T cells were from Stem Cell Technologies (USA).

2.2. Animals

Eight to ten-week-old inbred BALB/c mice and Swiss albino mice weighing approximately 20–25 g were used. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India regarding the maintenance and euthanasia of small animals were strictly followed.

2.3. Induction of lymphopenia

Animals were placed in ventilated perspex boxes and exposed to 600 cGy whole body γ -radiation at a dose of 420 cGy/min in a Gamma Cell-220 Irradiator (AECL, Canada). Lymphopenia induction was confirmed by counting the number of total splenic lymphocytes 48–96 h after irradiation (Sharma et al., 2007a).

2.4. In vitro cell proliferation

Spleen cell suspension was prepared as described earlier (Sharma et al., 2007a). Lymphocytes were stained with CFSE (20 μ M, 5 min, 37 °C) and washed three times using ice-cold RPMI medium containing 10% FBS (complete medium, CM). Percent daughter cells (showing a decrease in CFSE fluorescence intensity) were calculated using FloMax[®] software in a Partec PAS III flowcytometer.

2.5. Treatment with inhibitors and mitogens

Two million lymphocytes were stimulated with con A (10 μ g/ml) in presence of Gö6983 (25 nM)/PD98059 (50 μ M)/plumbagin (5 μ M)/rapamycin 10 nM + Gö6983 25 nM/Ly294002 20 μ M + PD98059 50 μ M and were cultured for 24 or 72 h at 37 °C.

2.6. Adoptive transfer and HDP in vivo

CD4⁺ T cells were purified from splenic lymphocytes using anti-CD4 coated magnetic nanoparticles in a MACS cell sorter. Cells containing >95% CD4⁺ population were stained with CFSE and cultured in presence of the inhibitors described in Section 2.5 for 16 h at 37 °C in 5% CO₂. In another group, CD4⁺ T cells were stained with CFSE and were incubated with plumbagin (5 μ M) for 4 h. In each group 1.5 million purified CD4⁺ T cells were injected intravenously or intraperitoneally into lymphopenic mice. At least four mice were used in each group. The reconstituted mice were sacrificed 96 h after injection and spleens and peritoneal exudate cells were recovered. Fifty thousand lymphocytes in each group were analyzed by flowcytometry to enumerate frequency of donor cells and to quantify the cell proliferation.

2.7. Visualization of donor T cells in the host spleen

Spleens from reconstituted lymphopenic mice and normal mice were frozen in liquid nitrogen and used for making 20 μ m sections using a cryostat. The sections were mounted on glass slides, fixed with para-formaldehyde and stained with PE conjugated anti-CD3 antibody.

2.8. Measurement of cytokine levels

Spleens from normal and reconstituted lymphopenic mice were harvested at different time intervals (4, 19 and 100 days) and the lymphocytes were stimulated with con A for 24 h. The levels of IL-2, IL-4, IL-6 and IFN- γ in the supernatants were estimated using commercial ELISA sets.

2.9. Western blotting

Total lymphocytes were isolated from the spleens of lymphopenic mice 19 days after reconstitution. The CD4⁺ T cells were sorted and lysed in cytosolic extraction buffer as described earlier (Sandur et al., 2006). The CD4⁺ T cells from normal mice served as control. Equal amount of protein was resolved using polyacrylamide gel electrophoresis and electro-blotted on nitrocellulose membranes and membranes were probed with primary antibodies for I κ B- α , pJNK, and pAKT followed by HRP-conjugated secondary antibody.

The membranes were stripped and reprobed with mouse actin- β antibody as a loading control.

2.10. Statistical analysis

The statistical significance of the differences between untreated cells and cells treated with different pharmacological inhibitors or plumbagin in respect of all parameters studied was assessed by Student's *t*-test.

3. Results

3.1. Cell division kinetics of MDP of T cells in vitro and HDP of CD4+ T cells in vivo

Fig. 1A shows representative dot plots of CFSE labeled lymphocytes stimulated with con A for 72 h in vitro as compared to that of unstimulated lymphocytes. The frequency of daughter cells increased significantly in con A stimulated cells (Fig. 1B) and majority of the dividing T cells showed lymphoblast phenotype as indicated by increase in size (forward scatter, Fig. 1A). Most of the T cells had undergone about 5 divisions during 72 h after stimulation with con A (Fig. 1A).

The labeled CD4+ T cells injected into normal syngeneic recipients did not divide. However, when injected into syngeneic lymphopenic mice, these cells divided (Fig. 1C, right panel) but did not form lymphoblasts (Fig. 1C right panel). Among the divided cells, majority of the cells (28%) had undergone only one division and about 15% cells had divided two times (Fig. 1D).

3.2. Spatial distribution of CD4+ T cells undergoing HDP

To decipher the specific anatomical locations that permit space sensing leading to HDP, the CFSE labeled CD4+ T cells were injected intravenously or intraperitoneally into lymphopenic or normal mice. Lymphocytes were recovered from the host spleen, lymph nodes or peritoneal fluid 96 h after injection. A significant increase in the fraction of donor derived daughter cells was seen in the spleen and lymph nodes of lymphopenic mice as compared to that from normal mice. On the contrary, there was no increase in the fraction of daughter cells in the peritoneal cavity of lymphopenic mice (Fig. 2A) indicating that T cells divided only in the secondary lymphoid organs of lymphopenic mice.

To further find out the spatial distribution of donor CD4+ T cells undergoing HDP within the host spleen, transverse sections were stained with PE-conjugated anti-CD3 antibody. T cell zones focally and avidly staining with CD3 antibody were visible in the spleen obtained from normal mice (Fig. 2B). The donor derived CFSE labeled T cells were distributed into these specific T cell zones in the host spleen (Fig. 2B and C).

3.3. Role of PI3kinase, AKT/mTOR, MAPkinase and PKC signaling pathways in MDP and HDP of T cells

Treatment of T cells with PD98059 or Gö6983 or a combination of Gö6983 + rapamycin or PD98059 + Ly294002 significantly inhibited the mitogen induced proliferation in vitro (Fig. 3A and B). However, the CFSE labeled donor CD4+ T cells treated with above inhibitors showed HDP comparable to that of untreated CD4+ T cells in lymphopenic mice (Fig. 3C and D). The frequency of donor cells was significantly reduced in the mice reconstituted with CD4+ T cells treated with the inhibitors which could be due to decrease in survival (Fig. 3E).

3.4. Involvement of NF- κ B and signaling mediators in lymphocytes during HDP

Fig. 4A shows degradation of I κ B- α and activation of pAKT and pJNK in CD4⁺ T lymphocytes isolated from lymphopenic mice 19 days after reconstitution. Actin- β levels were used as internal control. This suggested that NF- κ B, AKT and JNK were activated during HDP of CD4⁺ T cells.

3.5. Plumbagin inhibited HDP of CD4⁺ T cells

Fig. 4B shows the effect of plumbagin treatment (5 μ M, 4 h in vitro) on HDP of CD4⁺ T cells in autologous lymphopenic hosts. The histograms and dot-plots show donor derived CFSE⁺ CD4⁺ T cells in the host spleen 96 h after reconstitution (Fig. 4B). The frequency of plumbagin treated donor CD4⁺ T cells was 94% lower as compared to that of untreated CD4⁺ T cells injected into the lymphopenic mice (Fig. 4C). Thus plumbagin treated cells failed to survive or divide under lymphopenic conditions.

3.6. Cytokine production after HDP of CD4⁺ T cells in lymphopenic mice

Lymphopenic BALB/c mice were reconstituted with autologous lymphocytes isolated from age and sex matched donors and were sacrificed 4, 19 or 100 days after injection. The con A induced IL-2 production in cells from lymphopenic mice 4 and 19 days after reconstitution was significantly lower than that in con A treated lymphocytes from age and sex matched normal mice (Fig. 5A). However, IL-2 levels were significantly higher at 19 days as compared to 4 days after adoptive transfer. On the contrary, the IL-2 production in lymphocytes isolated from lymphopenic mice 100 days after adoptive transfer was similar to that in lymphocytes from normal mice (Fig. 5A). At 100 days, spleen size in mice recovering from lymphopenia was similar to that in normal mice indicating a complete recovery.

The con A induced IL-4 production in normal mice increased steadily with age indicating a progressive increase in Th2 bias in BALB/c mice. However, cells taken from lymphopenic mice 4 days after reconstitution and stimulated with con A produced significantly higher IL-4 than that in lymphocytes from normal mice. Interestingly, the IL-4 level decreased 19 and 100 days after reconstitution as compared to that in cells from normal mice (Fig. 5B).

The con A induced IL-6 production in lymphopenic mice 4 days after reconstitution was significantly lower than that in lymphocytes from normal mice (Fig. 5C). On the contrary, the IL-6 levels in lymphocytes from lymphopenic mice 19 and 100 days after reconstitution were significantly elevated as compared to that in cells from normal mice (Fig. 5C). These results indicated a clear augmentation of IL-6 production during recovery from lymphopenia.

The con A induced IFN- γ production in lymphopenic mice 4 days after reconstitution was significantly higher than that in lymphocytes from normal mice (Fig. 5D). The IFN- γ levels in lymphocytes from lymphopenic mice 19 days after reconstitution were lower than that in cells from normal mice. On the contrary, the IFN- γ production at 100 days after injection was again higher than that in lymphocytes from normal mice. BALB/c mice are known to have a genetic Th2 bias also reflected by an increase in con A induced IL-4 production with increased age in the present studies (Fig. 5B). Until recently, it was believed that IL-6 is produced by Th2 cells (Szabo et al., 2003). Thus the increased IL-6 production in lymphocytes during recovery from lymphopenia could be attributed to genetic Th2 bias in BALB/c mice. In order to dissect the contribution of inherent Th2 bias versus transient lymphopenia, these experiments were repeated using Swiss mice. The variation in levels of IL-2, IL-4 and IFN- γ during recovery from lymphopenia in Swiss mice was similar to that observed in BALB/c mice (Data not shown). Interestingly, con A induced IL-6 production in

lymphocytes from lymphopenic mice 4 and 19 days after reconstitution was significantly elevated as compared to that in cells from normal mice (Fig. 5E). These results clearly showed that recovery from lymphopenia was associated with an increased propensity of T cells to IL-6 production irrespective of their inherent Th1/Th2 bias.

3.7. Modulation of cytokine production by pharmacological inhibitors and plumbagin

Fig. 6 shows the effects of Gö6983 or PD98059 and their combinations with Ly294002 or rapamycin on con A induced cytokine production in lymphocytes. The lymphocytes treated with con A showed a significantly higher production of IL-2, IL-4, IL-6 and IFN- γ cytokines as compared to that in unstimulated cells. Gö6983 significantly inhibited con A induced IFN- γ production without inhibiting IL-2, IL-4 and IL-6 production (Fig. 6A–D). The combination of Gö6983 + rapamycin significantly inhibited IL-2, IL-4 and IFN- γ but not IL-6 production. PD98059 significantly inhibited IL-2 and IFN- γ production without inhibiting IL-4 and IL-6 production in con A stimulated lymphocytes (Fig. 6A–D). Interestingly, the cells treated with a combination of PD98059 + Ly294002 showed a significant inhibition of all the four cytokines (Fig. 6A–D). Plumbagin also suppressed con A induced IL-6 production in lymphocytes (Fig. 6E).

4. Discussion

Rocha et al. (1983) identified a novel space sensing-induced T cell proliferation in response to lymphopenia in vivo. This type of proliferation has been described by different authors as homeostatic proliferation or homeostasis driven proliferation (HDP) or lymphopenia induced proliferation. HDP has been shown to occur in T cells injected into thymectomized mice, irradiated lymphopenic mice as well as RAG deficient mice and required signaling through TCR (Rocha et al., 1983; Miller and Stutman, 1984; Freitas et al., 1986). It is proposed to be induced by low affinity self peptides similar to those driving their positive selection in thymus (Ernst et al., 1999; Sullivan et al., 2004). Recently, another form of HDP of T cells has been described as spontaneous proliferation characterized by fast, burst-like cell-cycle activity which may allow effector T cell differentiation (Min et al., 2005; Tajima et al., 2008).

Many common insults to the immune system may result in transient lymphopenia in vertebrate organisms, and are typically followed by uneventful immune reconstitution. However, in some clinical settings the drive to re-establish the T cell population size may compromise the diversity of the TCR repertoire and allow emergence of autoreactive clones, which in turn may lead to immunodeficiency and autoimmunity respectively (La Gruta et al., 2000; Theofilopoulos et al., 2001; Marleau and Sarvetnick, 2005). Thus, understanding the mechanisms that regulate T cell proliferation under lymphopenic conditions has important clinical applications.

In the present studies, MDP was compared with HDP of purified CD4⁺ T cells in irradiated lymphopenic mice. MDP of T cells was significantly faster as compared to HDP of T cells (Fig. 1). This observation is consistent with separate findings of others on MDP and HDP (Hagen et al., 2004; Kassiotis et al., 2003; Kieper et al., 2004; Quah et al., 2007). The slow division rate of HDP may ensure a tighter regulation of lymphocyte proliferation and continuous trafficking of dividing cells between different lymphoid organs via blood and lymph would ensure that “empty lymphoid space-sensing” occurs at the level of whole organism.

HDP was found to occur in the spleen as well as lymph nodes of the lymphopenic mice but not in peritoneum (Fig. 2). Several reports have shown the importance of lymphocyte trafficking through and homing to draining lymph nodes for antigen driven proliferation

using intravital microscopy (Mempel et al., 2004; Henrickson et al., 2008). Since peritoneal fluid is not directly connected to blood circulation, the factors sensing empty lymphoid space may not be accessible to the peritoneum resident lymphocytes. Antigen driven proliferation (ADP) is generally more robust in the draining lymph nodes and is localized to T cell zones (Mempel et al., 2004; Henrickson et al., 2008). Our results showed that HDP took place in lymph nodes as well as spleen and was localized to T cell zones in the later (Fig. 2) suggesting that HDP and ADP share common spatial requirements.

The present study confirmed that a broad spectrum PKC inhibitor, Gö6983, partially but significantly blocked the MDP of polyclonally activated T cells in vitro (Hermann-Kleiter et al., 2006). Treatment of CD4⁺ T cells with Gö6983 or MEK inhibitor PD98059 or a combination of Gö6983 plus rapamycin or PD98059 plus Ly294002 did not affect their HDP. The decreased frequency of inhibitor(s)-treated donor cells in the spleen of reconstituted lymphopenic mice indicated a possible role for PKC and MEK in survival of CD4⁺ T cells in lymphopenic mice (Figs. 3 and 4) (Kirberg et al., 1997; Pages et al., 1999).

Interestingly, the protein kinases AKT and JNK as well as proinflammatory transcription factor NF- κ B were activated in CD4⁺ T cells isolated from reconstituted lymphopenic mice (Fig. 4A). These proteins have also been shown to be activated during MDP of T cells (Baier et al., 1993; Agrawal et al., 2006). An essential role of NF- κ B for survival and HDP of CD4⁺ T cells was confirmed by the results showing severely impeded frequency of plumbagin treated CFSE labeled CD4⁺ donor T cells in the spleen of reconstituted lymphopenic mice (Fig. 4). Plumbagin has been shown to inhibit NF- κ B activation by prevention of I κ B- α degradation as well as by suppression of direct DNA binding ability of NF- κ B (Sandur et al., 2006). We have recently shown that plumbagin blocks LPS induced B cell proliferation by inhibition of NF- κ B activation (Rashmi et al., 2009).

Mitogen induced cytokine production in T cells recovered from reconstituted lymphopenic mice showed a pro-inflammatory pattern as compared to that in cells from normal mice. IL-2 and IFN- γ production showed steady increase during recovery from lymphopenia indicating that their levels may be used as reliable markers to assess the recovery from lymphopenia (Fig. 5A and D). Sanchez-Guajardo et al. (Sanchez-Guajardo et al., 2005) had shown that CD4⁺ Th2 cells from Stat4^{-/-} mice divided slower than CD4⁺ Th1 cells from Stat6^{-/-} mice. The present results using polyclonal CD4⁺ T cells showed a similar trend with increased IFN- γ production with time (Fig. 5D). However, a decrease in con A-induced IL-4 production in lymphocytes 19 and 100 days after reconstitution indicated that recovery from lymphopenia was possibly associated with decreased Th2 differentiation in mice (Figs. 4 and 5).

Most strikingly, mitogen induced IL-6 production was significantly higher in lymphocytes taken from lymphopenic BALB/c mice 19 and 100 days after reconstitution as compared to that in cells from age/sex matched normal mice (Fig. 5C). Recent evidence shows that IL-6 is produced primarily from TH17 cells (Langrish et al., 2005; Weaver et al., 2007). The decrease in a Th2 cytokine and a concurrent increase in a Th17 cytokine during recovery from lymphopenia may be an underlying cause for increased chances of autoimmunity in patients recovering from lymphopenia (Theofilopoulos et al., 2001; Baccala and Theofilopoulos, 2005; Calzascia et al., 2008). Hence, different pharmacological inhibitors were assessed for their ability to block IL-6 production in T cells. Gö6983, PD98059 and Gö6983 plus rapamycin failed to inhibit IL-6 production but affected IL-2, IL-4 and IFN- γ production to different extents (Fig. 6). Interestingly, plumbagin alone completely inhibited IL-6 production (Fig. 6E).

In summary, the present report describes the spatial distribution and signaling requirements for HDP and cytokine production by CD4⁺ T cells in lymphopenic environment. To our knowledge, this is the first report describing an elevated production of IL-6 in T cells taken from mice recovering from lymphopenia. For the first time a role for NF- κ B during survival and HDP of CD4⁺ T cells has been highlighted. The mechanistic basis for a possible use of plumbagin in inhibiting onset and progression of autoimmunity is indicated.

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Abbreviations

ADP	Antigen driven proliferation
CFSE	Carboxy fluorescein diacetate succinimidyl ester
Go	Gö6983
HDP	Homeostasis driven proliferation
Ly	Ly294002
MDP	Mitogen driven proliferation
PE	Phycoerythrin
PD	PD98059
Rapa	Rapamycin

Fig. 1.

Kinetics of mitogen driven proliferation of T cells in vitro vis a vis homeostasis driven proliferation (HDP) of CD4+ T cells in vivo: CD4+ T cells or total lymphocytes were stained with CFSE. Lymphocytes (2×10^6 cells) were stimulated with con A in vitro for 72 h. Alternatively, 1.5×10^6 CD4+ T cells were injected i.v. into tail vein of normal or lymphopenic mice. Lymphocytes were recovered from spleen of the host mice 96 h after reconstitution and 50,000 cells were acquired in FACS. (A) Representative dot plots and (B) percent daughter cells in each treatment group as estimated from decrease in mean fluorescence intensity. (C) Representative flowcytometric dot plots of CFSE labeled cells showing HDP of CD4+ T cells in lymphopenic mice. (D) The bars represent frequency of daughter cells in different division cycles in each treatment group. Data points represent mean $\pm$ S.E.M. for four mice and three such experiments were carried out. * $p < 0.01$, as compared to cells injected in normal mice. * $p < 0.01$, as compared to unstimulated cells.

Fig. 2.

HDP of CD4⁺ T cells in secondary lymphoid organs: Purified CD4⁺ T cells were stained with CFSE and 2×10^6 cells were injected i.v. or i.p. into normal and lymphopenic mice. Lymphocytes were recovered from spleen, lymph node and peritoneum of the host mice 96 h after injection and 50,000 cells were acquired in FACS. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (A) The bars represent percent daughter cells in the donor cell population isolated from different anatomical sites of the host. Data points represent mean $\pm$ S.E.M. for four mice and two such experiments were carried out. * $p < 0.01$, as compared to cells injected in normal mice. (B) T cell zones in a normal spleen: transverse sections (20 μ m) of fresh frozen spleen were mounted on glass slides and stained with PE conjugated CD3 antibody and observed under fluorescence microscope. Shown are the T cell zones in a spleen isolated from a normal mouse. Right panel shows higher magnification images of individual T cell zones. (C) T cells undergoing HDP formed T cell zones in spleen of a lymphopenic mouse. Purified CD4⁺ T cells from BALB/c mice were stained with CFSE and 2×10^6 cells were injected i.v. into lymphopenic BALB/c mice. Spleens from the host were harvested 96 h after injection and frozen in liquid nitrogen. Transverse sections (20 μ m) of fresh frozen spleen were mounted on glass slides and stained with PE conjugated CD3 antibody and observed under fluorescence microscope. Overlaid pictures show homing of the CFSE⁺ cells to the T cell zones of the host. Sections from two spleens are shown.

Fig. 3. Differential modulation of MDP and HDP of T cells by MEK inhibitor PD98059 or PKC inhibitor Gö6983 or their combination with rapamycin and PI3Kinase inhibitor Ly294002: Splenic lymphocytes were stained with CFSE and treated with PD98059 (50 μ M) or Gö6983 (25 nM) or PD98059 (50 μ M) + Ly294002 (20 μ M) or Gö6983 (25 nM) + rapamycin (10 nM) and were stimulated with Con A (10 μ g/ml) at 37 $^{\circ}$ C in CM in a 95% air/5% CO₂ atmosphere for 72 h. In each group 20,000 cells were acquired in FACS. (A) Representative flowcytometric histograms showing proliferation of T cells. (B) Purified CD4⁺ T cells were stained with CFSE and cultured in presence of PD98059 or Gö6983 or PD98059 + Ly294002 or Gö6983 + rapamycin overnight and 2×10^6 cells were injected i.v. into

lymphopenic mice. Lymphocytes were recovered from spleen of the host mice 96 h after injection and 50,000 cells were acquired in FACS. Representative flowcytometric histograms show proliferation of CFSE+ CD4+ T cells. (C) Percent daughter cells in response to con A in each treatment group. (D) The bars represent percentage of donor derived daughter cells in the spleen of lymphopenic host. (E) Frequency of donor cells in the host spleen. Data points represent mean $\pm$ S.E.M. from three replicates in vitro and four mice in vivo and two such experiments were carried out. $^{\#}p < 0.01$ as compared to Con A treated cells (MDP). $*p < 0.01$ as compared to untreated cells injected into lymphopenic mice.

Fig. 4.

Activation of NF- κ B during HDP of CD4⁺ T cells and modulation by plumbagin (A) Purified CD4⁺ T cells were injected i.v. into lymphopenic mice and were recovered from host spleen 4 days after injection. CD4⁺ T cells were sorted and used for analysis of IκB- α , phospho AKT, phospho JNK and actin- β using western blotting. (B) Purified CD4⁺ T cells were stained with CFSE and cultured in presence of plumbagin (5 μ M) for 4 h and 2×10^6 cells were injected i.v. into lymphopenic mice. Lymphocytes were recovered from spleen of the host mice 96 h after injection and 50,000 cells were acquired in FACS. Representative flowcytometric histograms and dot plots show proliferation of CFSE⁺ CD4⁺ T cells. (C) The bars represent frequency of donor cells in the host spleen. Data points represent mean $\pm$ S.E.M. from three replicates in vitro and four mice in vivo and two such experiments were carried out. * $p < 0.01$, as compared to that in untreated cells injected into lymphopenic mice.

Fig. 5.

Changes in cytokine production by lymphocytes during recovery from lymphopenia. Lymphopenic mice were reconstituted with autologous CD4⁺ T cells and sacrificed 4, 19 and 100 days after adoptive transfer. The splenic lymphocytes from mice recovering from lymphopenia and age/sex matched normal mice were cultured in presence of con A (10 μ g/ml) at 37 °C in CM in a 95% air/5% CO₂ atmosphere for 24 h. The levels of (A) IL-2, (B) IL-4, (C) IL-6 and (D) IFN- γ in the culture supernatants of cells obtained from BALB/c mice and (E) the levels of IL-6 in the culture supernatants of cells obtained from Swiss mice were estimated by ELISA. #*p* < 0.01, as compared to that in Con A stimulated cells from normal mice.

Fig. 6. Modulation of cytokine production by Gö6983, PD98059, Ly294002, rapamycin and plumbagin in Con A stimulated lymphocytes: Lymphocytes from BALB/c mice were treated with Gö6982 (25 nM) or PD98059 (50 μ M) or a combination of (Gö6983, 25 nM + Rapamycin 10 nM) or (PD98059, 50 μ M + Ly294002, 20 μ M). Another set of lymphocytes was treated with plumbagin (5 μ M) in RPMI medium free from 2-mercaptoethanol and 2×10^6 cells were stimulated with Con A (10 μ g/ml) at 37 °C in CM in a 95% air/5% CO₂ atmosphere for 24 h. Unstimulated cells were used as control. The levels of (A) IL-2, (B) IL-4, (C and E) IL-6 and (D) IFN- γ in the culture supernatant were estimated by ELISA. # p < 0.01, as compared to that in Con A treated cells.