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An *In vitro* Model for the Study of HIV Infection of Thymocytes

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Abstract

The study of human thymocytes requires an appropriate matrix to enable the proper function of thymocytes. Although OP9-DL1 cells have been well established as an ideal co-culture system for the generation of T-cells from their progenitors; their ability to support a mixed population of mature human thymocytes for *in vitro* HIV infection studies has yet to be established. We assessed the effects of co-culturing a heterogeneous population of mature human thymocytes with a mouse derived cell line (OP9) transduced with the notch ligand delta like 1 (OP9-DL-1) and compared this to standard co-culture with human thymic epithelial cells (TEC). Co-culturing thymocytes with OP9-DL1 cells resulted in higher viability and lower apoptosis when compared to TEC co-cultures. The subset distribution and CD127 expression of thymocytes varied slightly between conditions. Thymocytes co-cultured with OP9-DL1 cells had a lower proportion of CD3$^+$DP cells and higher proportion of SP4 cells compared to TEC co-cultures. The mature CD3$^+$CD4$^+$CD8$^-$ (SP4) cells also had lower levels of CD127 expression in OP9-DL1 cultures when compared to TEC. Interleukin-7 stimulation of thymocytes resulted in a decrease in CD127 expression in OP9-DL1 co-cultures, as previously observed with TEC co-cultures. Thymocytes co-cultured with OP9-DL1 tended to have higher levels of IL-7 induced STAT-5 phosphorylation and had higher levels of Interleukin-7 induced Bcl-2 expression. OP9-DL1 cells provide a microenvironment which is permissive to HIV infection in thymocytes in vitro. Co-culturing thymocytes with OP9-DL1 will facilitate the study of human thymocytes and aid in the study of exogenous stimuli or infection on individual thymocyte subsets.

Keywords: HIV; CD127; IL-7; OP9-DL1; Thymic epithelial cells; Thymocytes

Abbreviations: Bcl-2: B cell Lymphoma 2; DL-1: Delta Like 1; DP: Double Positive; ECD: Phycoerythrin-Texas Red; FITC: Fluorescein Isothiocyanate; FTOC: Fetal Thymic Organ Culture; HIV: Human Immunodeficiency Virus; HSC: Hemopoietic Stem Cells; ISP4+: Immature Single Positive CD4; PC5: PE-cyanin 5; PE: Phycoerythrin; PI: Propidium Iodide; SCF: Stem Cell Factor; SP: Single Positive; STAT: Signal Transducer and Activator of Transcription; TEC: Thymic Epithelial Cells; TN: Triple Negative

Introduction

The immune system is a highly specialized system that relies on the interplay of a large network of factors such as lymphoid organs, cells and molecular messengers (eg. chemokines and cytokines). Disrupting just one factor can result in altered immune regulation leading to immunodeficiency, autoimmunity or impaired T-cell development.

The thymus is a target organ of HIV-1 infection. Examination of the thymus of HIV+ pediatric patients reveals a loss of lymphoid cells, disruption of the cortico-medullary boundary and a general destruction of thymic architecture which may contribute to impaired immune regulation and altered immune reconstitution in HIV disease [1,2]. The exact mechanisms of HIV-1-induced thymic dysfunction have yet to be fully elucidated. Targets of HIV-1 within the thymus are primarily CD4-expressing thymocytes, however other cells such as macrophages, dendritic cells, and epithelial cells can also be infected [2-5]. Reduced thymic output following HIV-1 infection can be due to increased cell death of infected thymocytes or a decrease in immature thymocyte proliferation following HIV-1 infection [6,7]. Thymic function may also be disrupted at the positive/negative selection stages in development possibly due to altered responses to cytokine signals.

Thymocyte development strongly relies on the thymic environment. The architecture of the thymus is such that thymocytes receive different signals from the thymic stroma as they migrate through the thymus. The stroma delivers environmental cues which T-cells require in order to survive, proliferate and differentiate [8]. Interaction of T-cells with thymic epithelial cells (TEC) results in the release of a multitude of soluble factors such as interleukin-1 (IL-1), IL-6 and IL-7 which are required in T-cell development [9-12]. Due to such requirements, the study of thymocyte function *in vitro* has been highly dependent on the use of complex culture systems.

Evidence for the direct infection of thymocytes with HIV-1 has been provided with the use of fetal thymic organ cultures (FTOC), and in thymocyte/TEC suspension cultures. Thymocytes in these cultures are able to support high levels of HIV replication [13-16]. The ability of HIV to infect thymocytes *in vitro* was dependant on direct contact between thymocytes and TEC [4]. Although the FTOC has been proven to be an excellent system for the study of thymocytes, [17-19] the limitation of this system is the technical difficulties of establishing organ cultures, the requirement for mouse lobes although the source of thymocytes is human, as well as the high cost and manipulation associated with animal studies. The *in vitro* co-culture system of dispersed thymocytes and TEC requires the primary isolation of TEC which requires several days to achieve. The optimal *in vitro* co-culture system for the study of HIV infection of thymocytes would be a cell

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line that could provide similar signals to thymocytes than direct TEC contact resulting in infection.

A cell suspension model, developed by Zuniga-Pflücker, involving the OP9-DL1 culture system has been shown to allow the development of hemopoietic stem cells (HSC) into mature T-cells [20]. OP9 are a murine bone marrow derived stromal cell line, which expresses the notch ligand Delta like 1 (DL1). The advantage of this system is its simplicity and versatility. Although the system is based on a mouse cell line, the use of this co-culture system has been adapted to human cells and has been successful in supporting the development of human T-cells [21,22].

The OP9-DL1 cell has been developed to serve as a matrix for the study of thymocyte development, but its potential uses beyond this have not been evaluated. TEC/thymocyte interactions result in the release of soluble factors which in turn activate the thymocytes allowing for productive infection with HIV [4]. Determining if OP9-DL1 cultures can provide the appropriate survival and activation signals in order to study the effect of exogenous stimuli on mature thymocytes beyond the positive and negative stage of selection has yet to be evaluated. Here we compare co-culture systems of human thymocytes with either human TEC or OP9-DL-1 cells in order to determine if OP9-DL1 can serve as a matrix to support human thymocytes for the study of HIV infection on specific thymic subsets.

Materials and Methods

Thymocyte isolation

Thymic tissue was obtained during elective cardiac surgery at the Children's Hospital of Eastern Ontario (CHEO) with informed consent obtained prior to surgery. Thymocytes were isolated as previously described [23]. Briefly thymic tissue was cut into 1-3 mm^3 pieces with a scalpel and dispersed with the plunger of a 60 mm syringe prior to being separated on a Ficoll-Paque™ PLUS *(*Amersham Pharmacia, Piscataway, NJ*)* density gradient. The isolated cells were resuspended in McCoys 5A selective media (Invitrogen, Burlington, On) supplemented with 2 mM glutamine, 100 μ/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich Inc, Oakville Ont, Canada) and 10% fetal calf serum (FCS) (Cansera, Rexdale, Ont. Canada). Cell purity was assessed by flow cytometry. Freshly isolated thymocytes were stored at 4°C for 4-10 days as required prior to use as previously described [23].

Thymic epithelial cell isolation

TEC were isolated as previously described [23]. Briefly, the dispersed thymic tissue was digested in DNase/collagenase (Invitrogen, Burlington, Ont) (1500 units/ml) for 90 minutes at 37°C. The resulting fragments were then washed and platted with 7 ml of serum free media, D-MEM/F12 selective media supplemented with 100 υ/ml penicillin, 100 μg/ml streptomycin, 20 vg/ml epidermal growth factor, 10^{-9} M cholera toxin, insulin 3 υg/ml and transferin 10 μg/ml (all from Sigma-Aldrich, Oakville, Ont,). After 24-hour incubation media was replaced and cells were fed every 3-4 days in serum free media.

OP9-DL1 cells

OP9-DL1 cells were provided by Dr. Zuniga-Pflücker [20]. Cells were maintained in MEM-α (Invitrogen, Burlington, Ont, Canada) with 20% FCS and passaged every 3-4 days by trypsinization.

Thymocyte phenotype

The following fluorochrome labelled monoclonal antibodies were used CD3-ECD (clone UCHT1), CD4-FITC (clone 13B8.2), CD8-PC5 (clone B9.11), and CD127-PE (clone R34.34) (all from Beckman-Coulter, Missisauga, Ont, Canada). The distribution of the following developmental stages of T-cell maturation were evaluated following 96 hours of co-culture: (TN) CD3$^-$CD4$^-$CD8$^-$, (immature single positive CD4 ISP4$^+$) CD3$^-$CD4$^+$CD8$^-$, (DP) CD3$^{+/-}$CD4$^+$CD8$^+$ and (SP) CD4$^+$ or CD8$^+$ cells. The expression of CD127-PE (Beckman-Coulter, Missisauga, Ont, Canada) was measured on the various subsets at the onset of culture and overtime with stimulation with IL-7.

Thymocyte cultures

Thymocytes were either cultured alone or co-cultured with TEC or OP9-DL1 cells. Briefly 2x10^6 thymocytes/ 8 x10^4 TEC or OP9-DL1 were cultured in 2 ml of McCoys 5A selective media supplemented with 2 mM glutamine, 100 υ/ml penicillin, 100 and μg/ml streptomycin and 10% FCS in a 12 well plate for 24-96 hours at 37°C. For stimulation experiments thymocyte cultures were stimulated with IL-7 0-5000 πg/ml (R & D, Minneapolis, MN, 387 USA).

Apoptosis

Apoptosis was measured by annexin V/Propidium Iodide (PI) staining using a commercial kit from Molecular Probes, Invitrogen according to manufacturer protocol. Briefly bulk thymocytes were co-cultured for 96 hours with either OP9-DL1 or TEC. Subsequently 1x10^5 thymocytes were resuspended in annexin V binding buffer and stained with annexin V and PI in the dark at room temperature for 15 minutes. The cells were then diluted with 400 μl of binding buffer prior to analysis by flow cytometry.

IL-7 induced Intracellular pSTAT-5

To assess the effect of co-culture on the ability of thymocytes to respond to IL-7, STAT-5 phosphorylation in response to IL-7 was measured as previously described [24]. IL-7 induced STAT-5 phosphorylation was measured by stimulating thymocytes with IL-7 (R & D, Minneapolis, MN, 387 USA) at 1 vg/ml for 15 minutes at 37°C and 5% CO$_2$. The cells were prepared for intracellular staining by Caltag "fix and Perm" (Invitrogen, Burlington, Ont, Canada) reagents according to the manufacturer's protocol. Briefly the cells were fixed with reagent A for 15 minutes followed by 10 minute incubation in ice cold methanol in order to optimize the FITC signal prior to permeabilization with reagent B for 20 minutes and staining with pSTAT-5-FITC (Alexa Fluor 488 mouse antihuman STAT5 pY694, BD Biosciences, San Jose, CA, USA). All flow cytometry was conducted on a Epics Coulter Altra.

IL-7 induced Bcl-2 expression

To determine the effect of co-culture on thymocyte function, we measured Bcl-2 expression following IL-7 stimulus as previously described [24]. Briefly 1x10^5 thymocytes were stimulated with IL-7 1 vg/ml for 48 hours at 37°C and 5% CO$_2$ in the presence of either TEC or OP9-DL1 cells. The cells were prepared for intracellular staining by Caltag "fix and Perm" reagents following the manufacturer's protocol. Briefly the cells were fixed with reagent A for 15 minutes, washed and resuspended in permeabilization reagent B for 20 minutes. The cells were stained with Bcl-2-FITC (BD Biosciences, San Jose, CA, USA).

IL-7-induced glucose uptake

IL-7-induced glucose uptake was measured following the modified procedures of Swainson et al. [25]. Briefly Thymocytes were either cultured alone or co-cultured with TEC or OP9-DL1 cells. Thymocytes (2x10^6) with TEC (8x10^4) or OP9-DL1 (8x10^4) were cultured with

medium or IL-7 (10 vg/ml) for 96 hours at 37°C and 5% CO_2. Thymocytes (1x10⁵) were then washed and resuspended in 100 µl of Krebs/ringer buffer and incubated at 37°C for 30 minutes. Thymocytes were incubated with 2 µci of ³H-D-glucose (GE Healthcare, Piscataway, NJ) for 45 minutes at 37°C. Glucose uptake was terminated by the addition of ice cold Krebs/ringer buffer and washed 3 times to remove any residual glucose. The cells were solubilised in 0.1% SDS and radioactivity was measured by liquid scintillation counting on a Wallac MicroBeta TriLux (Perkin Elmer,Waltham,MA).

In vitro HIV-1 infection of thymocytes

Thymocytes were infected *in vitro* with a clinical isolate HIV$_{cs204}$ following previously described methods [26]. Briefly, prior to infection, thymocytes were resuspended to a concentration of 2x10⁶ cells/ml in McCoys 5A media with polybrene (Sigma-Aldrich) (3 µg/ml) for 1 hour at 37ºC. Thymocytes (6x10⁶) were then washed twice with PBS and the pellet was infected with cell-free HIV-1$_{cs204}$ supernatants at a multiplicity of infection (M.O.I) of 0.01 for 2 hours at 37ºC with shaking every 30 minutes or mock-infected with equivalent volumes of PBMC culture supernatants. Thymocytes were then washed twice in PBS and resuspended in McCoys 5A complete medium (Invitrogen).

PCR for the detection of HIV-1 in thymocytes

To confirm HIV infection of thymocytes, genomic DNA was isolated from infected thymocytes as early as 24 hours post infection (p.i.). Viral DNA was detectable by nested PCR targeting the gag region of HIV. Briefly, genomic DNA was isolated from infected thymocytes using the QIAGEN DNeasy blood and tissue kit (Qiagen, Mississauga, ON,). In the first round of PCR, DNA (1/10) was amplified with outer P24 primers (400 nm) fwd: 5'-ATAGAGGAAGAGCAAAACAAAA-3'; rvs: 5'-GTTCCTGAAGGGTACTAGTAGT-3'. The second round PCR used 5 µl of the product from the first round of PCR with inner p24 primers (400 nm) fwd 5'-CAAAATTACCCTATAGTGCA-3' and rvs 5'-ATGTCACTTCCCCTTGGTTCT-3'. Amplification conditions were as follows: 2 min at 95°C, (94°C for 60 s, 55°C for 60 s and 72°C for 60 s) for 30 cycles and 7 min at 72°C.

Statistical analysis

All statistical analyses and graphing were performed using GraphPad Prism 5.0 Software, (SanDiego, CA, USA). Statistical significance was determined by either Students t-test for paired samples or Anova with Dunnett post-test pairwise comparison. All flow cytometry was analysed using the FCS Express 2.0 software (De Novo Software, Thornhill, Ontario, Canada).

Results

Thymocytes co-cultured with OP9-DL1 cells are protected from spontaneous apoptosis

Within a co-culture system, contact with epithelial cells results in the release of soluble factors that are required for the viability of thymocytes. Thymocytes were cultured alone or co-cultured with either human TEC or OP9-DL1 cells for 96 hours and cell viability was assessed by trypan blue exclusion. Cell viability was decreased over time in thymocytes that were cultured alone (Figure 1A) indicating that thymocytes on their own (*i.e.* without co-culture) lack appropriate signals for survival. The cellular viability of thymocytes co-cultured with TEC only slightly decreased over time with 84 ± 3% of cells viable after 96 hours. The viability of thymocytes co-cultured with OP9-DL1 was also maintained with 92 ± 9% of thymocytes remaining viable after

96 hours of co-culture, indicating that both culture systems are able to provide the survival signals required for thymocytes in a short term culture. During the 96 hour culture there was a decrease in cell number in all culture conditions. This could possibly be due to the requirement for IL-7 and Flt3 ligand which is known to support differentiation of thymocyte in OP9-DL1 cultures [27-29]. The addition of IL-7 to either co-culture system resulted in higher levels of viability and increased the number of thymocytes in culture (data not shown). In order to confirm that the co-culture systems provide adequate survival signal, we measured apoptosis of the cells in culture. The level of spontaneous apoptosis as measured by annexin V⁺/ PI⁻ in thymocytes and after 96 hours was 56 ± 27% when cells were cultured alone and 57 ± 14% when thymocytes were co-cultured with TEC (Figure 1C). Co-culture with OP9-DL1 however resulted in protection from apoptosis with only 13 ± 5% of thymocytes staining annexin V⁺PI⁻, significantly lower than co-cultures with TEC (p= 0.009; Figure 1C). When the proportion of total annexin V⁺ cells was evaluated (annexin V⁺/ PI⁺ and annexin V⁺/ PI⁻) similar results were obtained.

The effect of co-culture on thymocyte phenotype

In order to determine if the subset distribution of total human
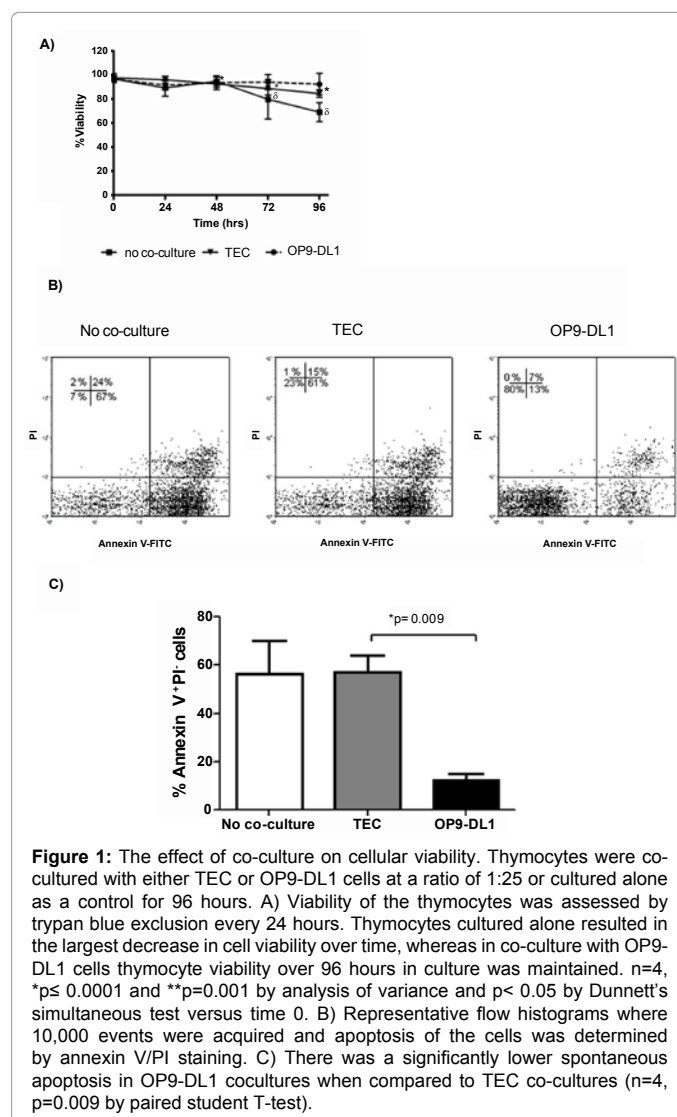

Figure 1: The effect of co-culture on cellular viability. Thymocytes were co-cultured with either TEC or OP9-DL1 cells at a ratio of 1:25 or cultured alone as a control for 96 hours. A) Viability of the thymocytes was assessed by trypan blue exclusion every 24 hours. Thymocytes cultured alone resulted in the largest decrease in cell viability over time, whereas in co-culture with OP9-DL1 cells thymocyte viability over 96 hours in culture was maintained. n=4, *p≤ 0.0001 and **p=0.001 by analysis of variance and p< 0.05 by Dunnett's simultaneous test versus time 0. B) Representative flow histograms where 10,000 events were acquired and apoptosis of the cells was determined by annexin V/PI staining. C) There was a significantly lower spontaneous apoptosis in OP9-DL1 cocultures when compared to TEC co-cultures (n=4, p=0.009 by paired student T-test).

thymocytes is altered in short term co-cultures, we measured the phenotype of thymocytes co-cultured with either TEC or OP9-DL1 for 96 hours. Short term co-culture with OP9-DL1 cells resulted in a lower proportion of cells in the CD3+DP subsets in comparison to TEC cultures, although this did not reach statistical significance. There were also a higher proportion of SP4 cells when compared to TEC co-cultures (12% *vs.* 29% respectively p=0.04; Figure 2B). Since IL-7 is critical for thymocyte function and the expression of its receptor is highly regulated on thymocytes, we measured the effect of co-culture on the expression of the IL-7 receptor (CD127). Co-culturing thymocytes with OP9-DL1 resulted in lower CD127 expression on SP4 cells when compare to TEC co-cultures (29.8 ± 14.5% vs. 51.8 ± 1.3%, p=0.05 respectively; Figure 2C). Culture conditions had no significant effect on CD127 expression on the other thymic subsets.

IL-7 downregulates CD127 expression on thymocytes in OP9-DL1 co-cultures

Since IL-7 is an important mediator of thymocyte differentiation and maturation, we measured the effect of co-culture on the ability of thymocytes to respond to IL-7. One established aspect of IL-7 activity is the downregulation of CD127 receptor expression on the surface

Figure 2: Effect of co-culture on thymocyte phenotype. Thymocytes were co-cultured with TEC or OP9-DL1 for 96 hours. A) Representative figure of subset distribution of total thymocytes where 10,000 events were acquired. B) OP9-DL1 co-culture resulted in a trend towards a lower proportion of CD3+DP (p=0.07) compared to TEC and a statistically significant higher proportion in SP4 cells (p=0.04) when compared to TEC cultures. n=4 p=0.04 by student t-test C) CD127 expression on individual subsets was determined by staining total thymocytes and gating on individual subsets. Thymocytes co-cultured with OP9-DL1 cells had a lower level of CD127 expression on SP4 cells when compared to TEC co-cultures. (n=4 p=0.05 Student t-test).

of T-cells [23]. To determine the effect of OP9-DL1 co-culture on IL-7 regulation of the CD127 receptor we measured the effect of IL-7 stimulation of thymocytes in OP9-DL1 co-cultures. IL-7 stimulation resulted in a rapid decrease in CD127 on mature thymocytes (Figure 3). The decrease was similar to what has been previously reported for thymocytes co-cultured with TEC [23]. At higher concentrations of IL-7 (5000 πg/ml) the decrease in CD127 expression was maintained through 96 hours in DP, SP4 and SP8 thymic subsets. The kinetics of CD127 decrease varied with the concentration of IL-7. At the lower concentration of 100 πg/ml of IL-7 the decrease in CD127 was transient with a recovery of CD127 receptor expression observed by 48 hours (Figure 3).

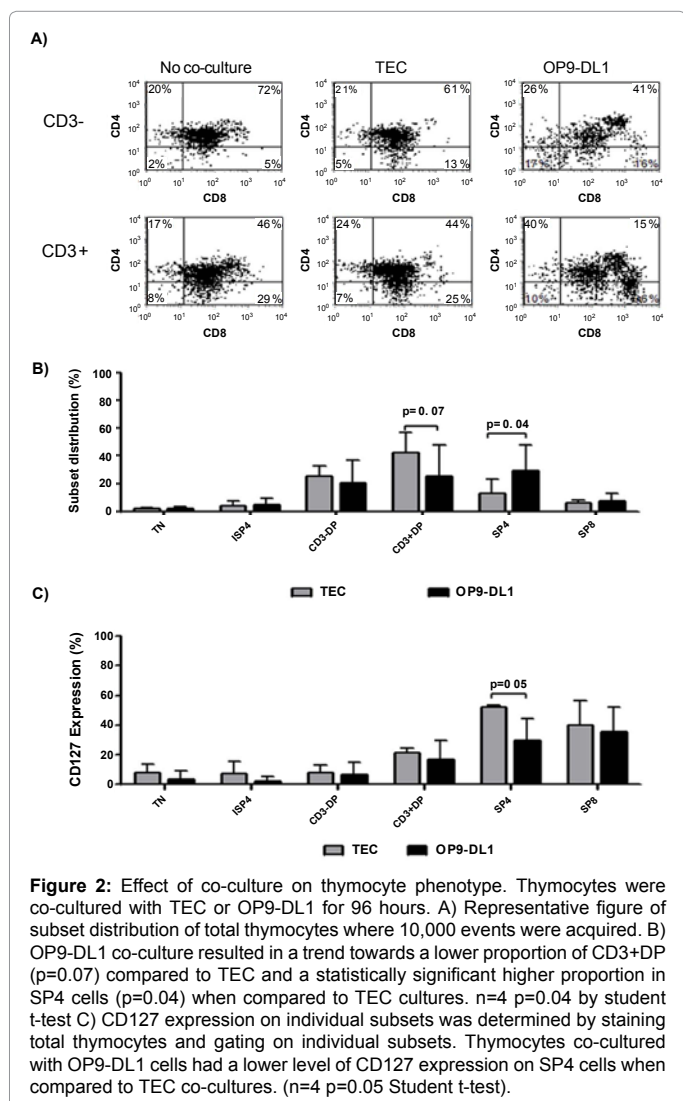### Co-culture is required to maintain IL-7 responsiveness of thymocytes

To determine if human thymocytes in co-culture remained functional *ex vivo*, we co-cultured thymocytes with TEC or OP9-DL1 and measured IL-7 responsiveness. IL-7 stimulation did not induce phosphorylation of STAT-5 in thymocytes when cultured alone (Figure 4). Thymocytes that were co-cultured with OP9-DL1 had a significantly higher level of basal pSTAT-5 when compared to thymocytes co-cultured with TEC, as measured by the percentage of pSTAT-5+ cells (5.3 ± 1.8% vs. 1.0 ± 0.4%; p=0.007). IL-7 stimulation of thymocytes resulted in a statistically significant increase in pSTAT-5 (Figure 4), which is consistent with previous reports [30-32]. Thymocytes that were co-cultured with OP9-DL1 cells tended to exhibit a greater degree of Stat-5 phosphorylation in response to IL-7 than thymocytes co-cultured with TEC (OP9-DL1: 17.4 ± 5.8% vs. TEC: 11.3 ± 1.1%; p=0.07), although this did not reach statistical significance. However the absolute increase in pSTAT-5 induced by IL-7 was similar in both co-culture conditions (OP9-DL1: 10.2% ± 1.4% increase and TEC: 12.1% ± 6.6% increase).
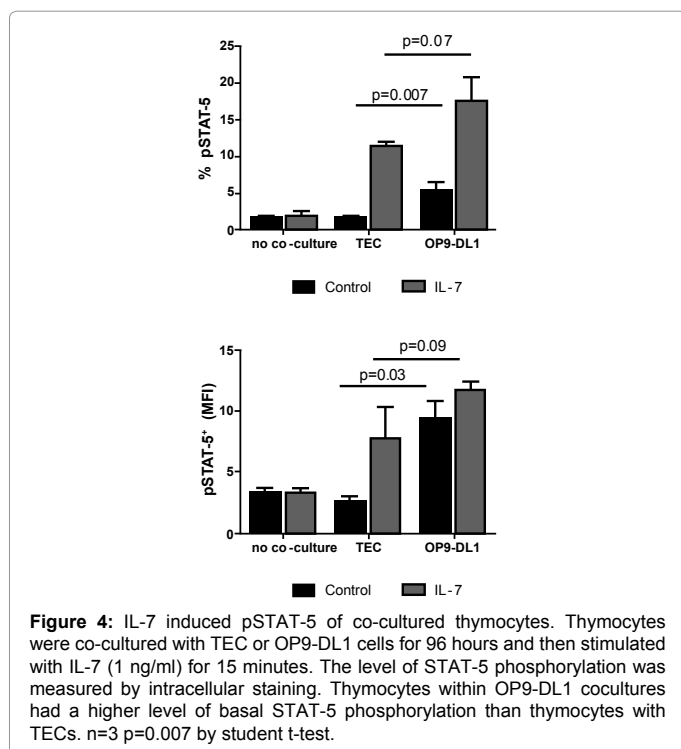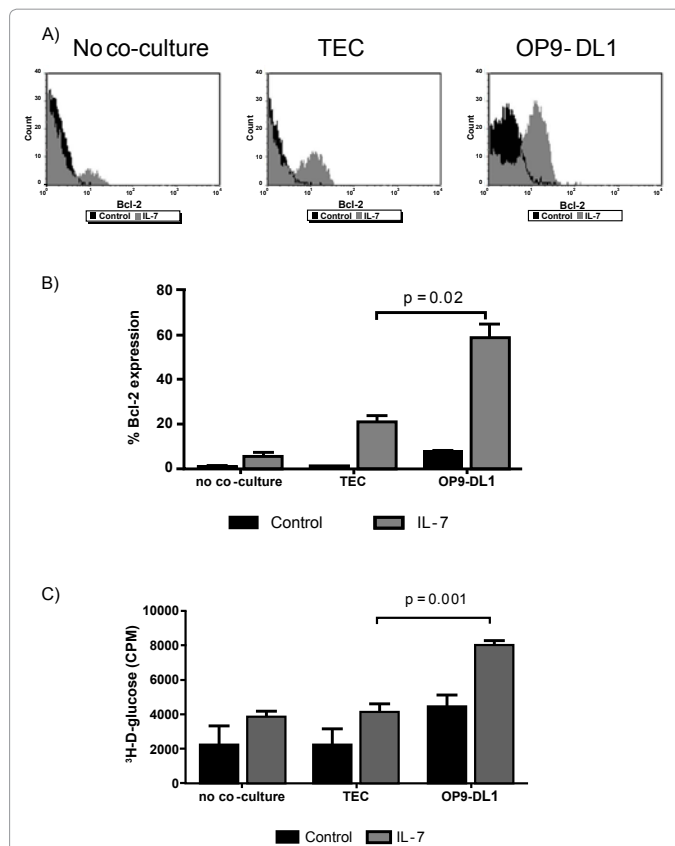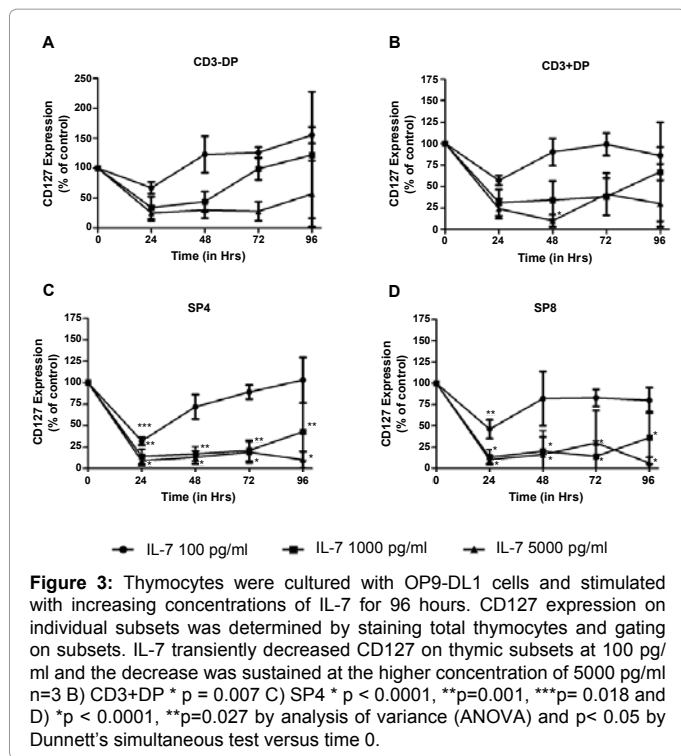
IL-7-induced Bcl-2 expression is enhanced in thymocytes co-cultured with OP9-DL1 cells

IL-7 has been reported to induce Bcl-2 expression in thymocytes [32,33]. IL-7 had little effect on Bcl-2 expression in thymocytes cultured alone (Figure 5). We observed a higher level of basal Bcl-2 expression in bulk thymocytes following 96 hours of culture in OP9-DL1 co-cultures when compared to TEC co-cultures as measured by the percentage of cells expressing Bcl-2 (TEC: 1.0 ± 0.0% vs. OP9-DL1: 7.0 ± 1.7%; p=0.002). IL-7 stimulation resulted in a higher percentage of cell expressing Bcl-2 in thymocytes co-cultured with OP9-DL1 when compared to thymocytes co-cultured with TEC (59.0 ± 10.4% vs. 20.3 ± 6.1%; Figure 5B p=0.003). The absolute increase in Bcl-2 expression following IL-7 stimulation of thymocytes was also significantly higher in thymocytes co-cultured with OP9-DL1 then with thymocytes co-cultured with TEC (52.0 % ± 9.8% vs. 19.3 % ± 6.1%; P=0.012).

IL-7-induced glucose uptake is enhanced in thymocytes co-cultured with OP9-DL1 cells

To determine the effect of IL-7 on metabolic processes, IL-7-induced glucose uptake in thymocytes was measured. IL-7 has been demonstrated to induce glucose uptake in recent thymic emigrants and circulating T-cells [25,34]. Thymocytes were either cultured alone or co-cultured with TEC or OP9-DL1 cells and stimulated with IL-7 (10 vg/ml) for 96 hours (Figure 5C). Thymocytes that were co-cultured with OP9-DL1 cells tended to have a higher level of basal glucose uptake when compared to thymocytes co-cultured with TEC, however this difference did not reach statistical significance (OP9-DL1:

Figure 3: Thymocytes were cultured with OP9-DL1 cells and stimulated with increasing concentrations of IL-7 for 96 hours. CD127 expression on individual subsets was determined by staining total thymocytes and gating on subsets. IL-7 transiently decreased CD127 on thymic subsets at 100 pg/ml and the decrease was sustained at the higher concentration of 5000 pg/ml n=3 B) CD3+DP * p = 0.007 C) SP4 * p < 0.0001, **p=0.001, ***p= 0.018 and D) *p < 0.0001, **p=0.027 by analysis of variance (ANOVA) and p< 0.05 by Dunnett's simultaneous test versus time 0.

Figure 4: IL-7 induced pSTAT-5 of co-cultured thymocytes. Thymocytes were co-cultured with TEC or OP9-DL1 cells for 96 hours and then stimulated with IL-7 (1 ng/ml) for 15 minutes. The level of STAT-5 phosphorylation was measured by intracellular staining. Thymocytes within OP9-DL1 cocultures had a higher level of basal STAT-5 phosphorylation than thymocytes with TECs. n=3 p=0.007 by student t-test.

4401 ± 1333 cpm vs. TEC: 2163 ± 1665 cpm; p=0.07). Thymocytes that were co-cultured with OP9-DL1 had greater levels of glucose uptake following IL-7 stimulation then thymocytes co-cultured with TEC (OP9-DL1: 8044 ± 345 cpm vs. TEC: 4144 ± 941 cpm; p=0.001). Although thymocytes co-cultured with OP9-DL1 cells had higher levels of IL-7-induced glucose uptake the absolute increase in glucose uptake following IL-7 was similar in both culture systems.
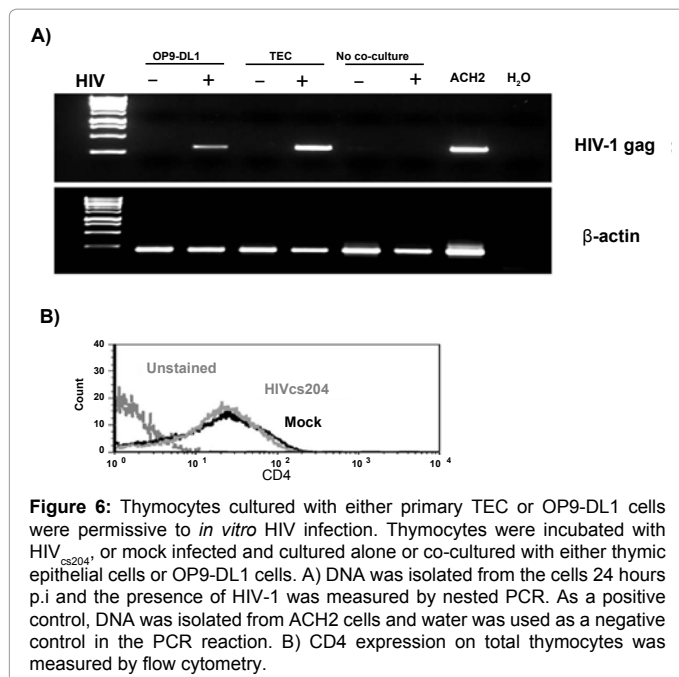

Figure 5: IL-7 enhances thymocytes survival of co-cultured thymocytes. A) Thymocytes were cocultured with TEC or OP9-DL1 cells for 96 hours and then were stimulated with IL-7 (1 ng/ml) for 48 hours. Bcl-2 expression was measured by intracellular staining. Thymocytes co-cultured with OP9-DL1 had a significantly higher level of Bcl-2 expression in response to IL-7 than thymocytes cocultured with TEC. n=4 p=0.003 by student t-test. B) Thymocytes were co-cultured with TEC or OP9-DL1 cells and stimulated with IL-7 for 96 hours. Glucose uptake was measured in the thymocytes by 3H-D-glucose. Thymocytes co-cultured with OP9-DL1 had a significantly higher level of glucose uptake in response to IL-7 than thymocytes co-cultured with TEC. n=3 p=0.007 by student t-test.

Thymocytes co-cultured with OP9-DL1 cells are infected by HIV-1

Thymocytes were infected *in vitro* at an m.o.i of 0.01 with a dual tropic strain of HIV and cultured alone or co-cultured with either OP9-DL1 or TEC as co-culture conditions provide the required stimulus for thymocytes to be infected [4,14]. CD4 expressing thymocytes are the main targets of HIV and have been shown to express both CXCR4 and CCR5 [35,36]. To demonstrate infection of thymocytes, the presence of HIV-1 DNA in thymocytes was measured by nested PCR. HIV infected thymocytes cultured alone had no detectable gag DNA (Figure 6B) confirming the requirement for cell contact with TEC for HIV infection *in vitro* [4,14]. HIV infected thymocytes that were co-cultured with either TEC or OP9-DL1 cells had detectable viral DNA (Figure 6A). HIV infection did not affect the overall expression of CD4 on thymocytes (Figure 6B). This is the first time that OP9-DL1 cells have been shown to provide the appropriate microenvironment for HIV infection of thymocytes.

Discussion

T-cell development requires a specialized microenvironment that

Figure 6: Thymocytes cultured with either primary TEC or OP9-DL1 cells were permissive to *in vitro* HIV infection. Thymocytes were incubated with HIV$_{cs204}$, or mock infected and cultured alone or co-cultured with either thymic epithelial cells or OP9-DL1 cells. A) DNA was isolated from the cells 24 hours p.i and the presence of HIV-1 was measured by nested PCR. As a positive control, DNA was isolated from ACH2 cells and water was used as a negative control in the PCR reaction. B) CD4 expression on total thymocytes was measured by flow cytometry.

is mainly provided by the thymic stroma [37]. A number of culture systems have been developed to study T-cell development *in vitro*, however they primarily involve work with mice. The OP9 murine cells were first used to support the development of B-cells; however the cell line would not support T-cell development, possibly because it did not provide notch signalling which is required for T-cell commitment and development. The OP9-DL1 and OP9-DL4 culture systems, which provide notch signalling, have been widely used for the study of commitment of precursors cells and subsequent T-cell development [20]. Although the system is based on a mouse stromal cell line it has been demonstrated to support the development of human hematopoietic stem cells, and human cord blood in long term cultures [21,22].

To understand the potential role that exogenous stimuli or viral infection may play in thymic dysfunction there is a requirement for an *in vitro* culture system that can support a heterogeneous population of human thymocytes. It has been previously established that contact with TEC is required in order to maintain thymocyte viability and allow permissiveness for *in vitro* HIV infection [4]. In this study we evaluated the capability of OP9-DL1 cells to support the function of human thymocytes and allow for the permissiveness for *in vitro* HIV infection.

Our results confirmed the importance of a co-culture system for functional thymocyte studies since thymocytes cultured alone exhibited significantly reduced viability and responded poorly to IL-7 (Figure 1, Figure 4, Figure 5). Several factors are released upon co-culture of thymocytes with TEC that play a role in thymocyte viability, proliferation and activation. Such factors include hormones such as thymulin [38-40] and cytokines, including IL-1, IL-6, and IL-7 [41]. The viability of thymocytes was the greatest with OP9-DL1 co-culture; therefore they appear to be a good candidate for co-culture with a heterogeneous population of thymocytes.

Co-culture with OP9-DL1 cells protects thymocytes from apoptosis (Figure 1C). *In vivo* the majority of thymocytes are undergoing apoptosis due to the selection process. Thymocytes studied *ex-vivo* may

have already received certain signals and have entered the apoptotic pathway prior to isolation and co-culture. Notch signalling leads to protection from T-cell receptor (TCR)-induced apoptosis as well as glucocorticoid induced apoptosis, hence thymocytes co-cultured with OP9-DL1 may be expected to have reduced apoptosis [42-45].

To compare the co-culture systems we determined the effect of the co-culture systems on cell subset distribution of a heterogeneous population of thymocytes in a short term culture. Thymocyte subset distribution was slightly different within the OP9-DL1 co-culture when compared to co-culture with TEC. The proportion of CD3$^+$DP cells was lower, while the proportion of SP4 cells was higher in thymocytes that were co-cultured with OP9-DL1 cells. Since the maturation and development of T-cells requires long term culture [5], the effect on phenotype that we observed within 96 hours is probably not due to maturation of cells from CD3$^+$DP to SP4 cells. One possible explanation could be decreased proliferation in the CD3$^+$DP subset resulting in an alteration in the proportion of cells within the other subsets of thymocytes co-cultured with OP9-DL1. The observed difference between the two culture systems is probably not due to differences in notch signalling since TEC also express notch ligands such as jagged-1, jagged 2 and DL1 [46].

The expression of CD127 is highly regulated in thymocytes [47]. Our data revealed a lower level of CD127 expression on SP4 cells within OP9-DL1 cultures when compared to TEC. Multiple cytokines have regulatory effect on the expression of CD127. IL-2, IL-4 and IL-7 stimulation results in decreased CD127 expression on the surface of both thymocytes and mature CD4$^+$ and CD8$^+$ T-cells [23,24,48-51]. Therefore, the lower levels of CD127 on SP4 cells may be due to cytokines produced OP9-DL1/thymocyte cultures. In fact, mean CD127 expression was lower in all subsets within the OP9-DL1 co-culture system, but this difference only reached statistical significance within the SP4 subset. Both OP9-DL1 and human TECs are capable of producing IL-7 [52]. However, the level of IL-7 produced within these systems is likely negligible since co-culture alone did not affect CD127 expression on SP8 cells (Figure 2) and we have shown that as little as 100 πg/ml of IL-7 can significantly reduce CD127 expression on SP8 cells (Figure 3). Therefore, the amount of endogenous IL-7 is too low to affect the outcome of our assays.

As a gauge of IL-7 function, we measured the effect of IL-7 stimulation on CD127 expression within an OP9-DL1 co-culture. We were able to demonstrate that human thymocytes in OP9-DL1 co-cultures were able to respond to IL-7, as measured by CD127 down regulation and that this response was greater than previously reported with TEC co-cultures [23]. This suggests that co-culture with OP9-DL1 may enhance IL-7 responsiveness of thymocytes.

We again demonstrated the importance of using a co-culture system in studies of thymocyte function since thymocytes that are cultured without the benefit of co-culture are unresponsive to IL-7 stimulation as measured by STAT-5 phosphorylation and Bcl-2 induction. Interactions between TEC or OP9-DL1 cells and thymocytes were sufficient for thymocytes to remain responsive to IL-7. Thymocytes co-cultured with OP9-DL1 cells had higher levels of basal STAT-5 phosphorylation than thymocytes co-cultured with TECs. This may be due to soluble factors that are released following thymocyte/OP9-DL1 cell interactions which lead to cell activation and hence STAT-5 phosphorylation. Although the level of STAT-5 phosphorylation following IL-7 stimulation was higher in OP9-DL1 cultures, the absolute change in STAT-5 phosphorylation following stimulation was similar in the two co-culture systems. Therefore, the

higher level of STAT-5 phosphorylation in OP9-DL1 cultures may be due to the activation state of the thymocytes rather than a specific increase in IL-7 responsiveness.

Given its role in the apoptotic pathway, the level of basal Bcl-2 expression could be indicative of the propensity of the thymocytes towards cell survival. We demonstrated that co-culture of thymocytes with OP9-DL1 cells resulted in higher level of Bcl-2 expression when compared to cells cultured with TEC or cultured alone. Bcl-2 is upregualted in thymocytes in order to protect cells from apoptosis [53,54] and notch signalling leads to increases in Bcl-2 expression in thymocytes [55]. Hence the decrease in apoptosis that was seen in co-culture with OP9-DL1 may be due to this increased level of Bcl-2. Our data also demonstrated higher levels of glucose uptake in thymocytes co-cultured with OP9-DL1 cells. Increases in glucose metabolism are one way in which cells regulate survival [34,56] and notch signalling cooperates with IL-7 to increase proliferation of immature thymocytes, glucose metabolism and Bcl-2 induction [57-59].

Through the evaluation of these co-culture systems on IL-7 activity, we demonstrated that IL-7 activity, as measured by Bcl-2 induction and glucose uptake, was greater in thymocytes in OP9-DL1 co-culture than those in TEC cultures, indicating that OP9-DL1 cells create a milieu in which thymocytes are more responsive to IL-7.

Although the use of OP9-DL1 cells as a support system for the generation of CD4+ and CD8+ T-cells from progenitor cells *in vitro* has been well established we demonstrate for the first time that OP9-DL1 cells are able to support the function and responsiveness of a heterogeneous population of mature human thymocytes, as well as provide the necessary signals that are required for *in vitro* infection with HIV. Our data clearly show that co-culture of human thymocytes with OP9-DL1 cells result in greater IL-7 responsiveness than co-cultures with human TEC. This demonstrates that they are an appropriate candidate for further studies of the effect of exogenous stimuli on thymocyte function. Although the backbone of the OP9-DL1 system is a murine cell, the co-culture was able to support human thymocytes and in fact was associated with greater viability of human thymocytes. The use of OP9-DL1 cells will provide a useful tool for future studies on the effect of HIV infection on the function of mature human thymocytes.

Acknowledgements

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