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Ageing impairs the T cell response to dendritic cells

Subtitle: immune system and ageing

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Abbreviations

CCR	C-C chemokine receptor type
DCs	dendritic cells
FCS	foetal calf serum
FoxP3	forkhead box P3
LDC	low density cells
LPS	lipopolysaccharide
MLR	mixed leucocyte reaction
mDCs	myeloid DCs
PBMC	peripheral blood mononuclear cell
pDCs	plasmacytoid DCs
TLR	toll-like receptor
Treg	regulatory T cells

Abstract

Dendritic cells (DCs) are critical in priming adaptive T-cell responses, but the effects of ageing on interactions between DCs and T cells are unclear. This study investigated the influence of ageing on the maturation of and cytokine production by human blood-enriched DCs, and the impact on T cell responses in an allogeneic mixed leucocyte reaction (MLR). DCs from old subjects (65-75y) produced significantly less TNF- α and IFN- γ than young subjects (20-30y) in response to lipopolysaccharide (LPS), but expression of maturation markers and co-stimulatory molecules was preserved. In the MLR, DCs from older subjects induced significantly restricted proliferation of young T cells, activation of CD8⁺ T cells and expression of IL-12 and IFN- γ in T cells compared with young DCs. T cells from older subjects responded more weakly to DC stimulation compared with young T cells, regardless of whether the DCs were derived from young or older subjects. In conclusion, the capacity of DCs to induce T cell activation is significantly impaired by ageing.

1. Introduction

Immunosenescence involves a progressive decline in immune function, which is associated with increased susceptibility to infections and poor response to vaccination. Many aspects of adaptive immunity are affected by ageing (Dunn-Walters, Banerjee et al. 2003; Swain, Clise-Dwyer et al. 2005; Frasca, Landin et al. 2008) . DCs play a central role in the priming of adaptive immune responses, but the influence of ageing on DC function remains poorly understood. Some studies demonstrate reduced numbers of both plasmacytoid and myeloid DCs (pDCs and mDCs) in the peripheral blood of older subjects (Shodell and Siegal 2002; Della Bella, Bierti et al. 2007; Perez-Cabezas, Naranjo-Gomez et al. 2007), but there is inconsistent data regarding the effects of ageing on DC phenotype and the ability of DCs to function as antigen presenting cells (Agrawal and Gupta 2011; Solana, Tarazona et al. 2012).

Induction of T cell responses by DCs involves the binding of antigen with major histocompatibility complex, DC expression of surface co-stimulatory molecules (e.g. CD40, CD80 and CD86), interaction with ligands on the T cell surface and production of cytokines. The efficacy of DCs in inducing T cell responses depends on a number of factors; impaired maturation of DCs, or altered cytokine production are likely to have a knock-on effect on the T-cells that they stimulate. To date, data regarding the influence of ageing on human DC expression of co-stimulatory molecules and cytokine production, in response to *in vitro* maturation-inducing stimuli, has been inconsistent, showing either comparable or reduced DC function in the elderly (Lung, Saurwein-Teissl et al. 2000; Pietschmann, Hahn et al. 2000; Saurwein-Teissl, Romani et al. 2000; Shurin, Shurin et al. 2007).

It is not clear whether DCs from young subjects are able to overcome the impaired responsiveness of T cells from older subjects and human studies investigating this are very limited (Agrawal, Agrawal et al. 2007; Jiang, Fisher et al. 2011). Lung, Saurwein-Teissl et al. (2000) demonstrated that human monocyte-derived DCs from young and older subjects were

equally effective in stimulating proliferation of and cytokine secretion by influenza-specific CD4⁺ T cells. In contrast, Agrawal, Agrawal et al. (2007) demonstrated that monocyte-derived DCs from older people were defective in stimulating proliferation of CD4⁺ naïve T cells from young subjects.

The aim of the present study was to investigate the influence of ageing on the maturation of and cytokine production by human blood-enriched DCs, and to investigate whether these age-induced alterations have an impact on DC and T cell responses in the MLR. By avoiding the use of cell lines and long cultures with addition of cytokines and growth factors, this represents a novel approach to study the influence of ageing on both DC and T cell function.

2. Materials and Methods

2.1. Peripheral blood mononuclear cell (PBMC) preparation and culture

Peripheral blood was obtained from healthy young (20-30y) and older (65-75y) subjects. Exclusion criteria included diabetes requiring medication, asplenia and other acquired or congenital immunodeficiencies, any autoimmune disease, malignancy, cirrhosis, connective tissue diseases; current use of immunomodulating medication (including oral prednisone and inhaled steroids), self-reported symptoms of acute or recent infection, use of antibiotics within last 3 months, alcoholism and drug misuse (University of Reading Ethics Committee project ref 10/05). Blood was diluted into an equal volume of RPMI 1640 medium. PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (Fisher Scientific, UK), and resuspended in RPMI 1640 medium with 10% foetal calf serum (FCS, Sigma Ltd, UK). PBMCs were cultured overnight in culture flasks (2×10^7 cells/flask) in a 37°C, 5% CO₂ atmosphere.

2.2. Human blood DC enrichment and culture

Low Density Cells (LDCs) were prepared as the source of human blood-enriched DCs (which normally represent 1-2% of PBMCs). The LDCs had morphological characteristics of DCs, as described in previous studies (Knight, Farrant et al. 1986; Kerdiles, Stone et al. 2010), typically 98-100% HLA-DR positive and stimulate strong proliferation of allogeneic T-cells at very low concentrations (Knight, Farrant et al. 1986; Holden, Bedford et al. 2008). Since DCs are unique in their ability to generate primary T-cell responses, T-cell responses generated in the MLR were interpreted as a result of DC stimulation. Unlike monocyte-derived DCs, homing markers are not altered during culture of LDCs, which is an advantage (Mann, Bernardo et al. 2012).

After overnight culture of PBMC, the non-adherent cells were collected and centrifuged over Nycoprep (500 x g, 15min) (PROGEN Biotechnik GmbH). LDCs were removed from the interface, washed twice (650 x g, 5 min) in RPMI 1640 medium with 10% FCS and resuspended in the same medium.

LDCs were adjusted to a concentration of 1×10^6 cells/ml and cultured in the presence or absence of 10 µg/ml LPS in a 37°C, 5% CO₂ atmosphere for 24h (see Fig. 1).

2.3. T cell purification and DC stimulation of T cells

In this allogeneic MLR, T cells were obtained from blood donated by healthy young or older subjects, which were different from the DC donors. After isolating PBMCs, T cells were separated by negative isolation using a Human T cell enrichment kit (BD Bioscience, UK). The purity of T cells after this selection process was higher than 98%, as determined by CD3 antibody staining. Prior to culture, purified T cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Ltd, UK) for subsequent assessment of T cell proliferation. T cells (4×10^5) from young and older subjects were co-incubated with 3% cultured LDCs from young and older subjects in a 37°C, 5% CO₂ atmosphere for 5d, using the experimental design illustrated in Fig. 1.

2.4. Intracellular cytokine production

Intracellular cytokine production by DCs following 24h incubation in the presence or absence of LPS, or by T cells following the MLR was analysed using flow cytometry. Cells were incubated with or without 50µl Monensin (3µM) (eBioscience Ltd, UK) in a 37°C, 5% CO₂ atmosphere for 4h, washed in FACS buffer (BD Bioscience, UK) and stained with the appropriate surface marker antibodies before fixation, permeabilisation and staining with appropriate antibodies for intracellular cytokines.

2.5. Antibody staining

For identification and characterization of peripheral blood DCs, cultured LDCs were stained with HLA-DR (APC, PerCP-Cy 5.5 or PE) and with a lineage cocktail containing antiCD3, CD14, CD19, CD20 (FITC). Cells that were negative to the cocktail but positive to HLA-DR were identified as DCs (see Fig. 2A). On average, 93% of LDCs were Lin⁻HLA-DR⁺. This was used in conjunction with antibodies for maturation markers (CD80 (PE-Cy 7), CD86 (APC) and CD40 (APC-Cy 7)), or a migration marker, C-C chemokine receptor type7 (CCR7) (PerCP-Cy 5.5). Cultured T cells were identified by CD3 (PE-Cy 7, APC or APC-Cy 7) staining (see Fig. 2B) and classified into CD4 (PE-Cy 7) and CD8 (PerCP-Cy 5.5) subsets. CD25 (APC) was used as a marker for T cell activation; and integrin β7 (PE) as a homing marker. Antibodies against IL-10 (PE), IL-12 (PE), TNF-α (PerCP-Cy 5.5), IFN-γ (APC-Cy 7) and TGF-β (PE-Cy 7) were used to assess intracellular cytokine production by Lin⁻HLA-DR⁺ DCs or DC-stimulated CD3⁺ T cells. Isotype-matched control antibodies included rat IgG2a (PE), mouse IgG1 (FITC, PE, PE-Cy 7, PerCP-Cy 5.5, APC-Cy 7), mouse IgG2a (PerCP-Cy 5.5) and mouse IgG2b (PerCP-Cy 5.5). Stained cells were incubated at room temperature in the dark for 30-45min, washed twice, resuspended in Fix solution and kept at 4°C until analysis by flow cytometry. The lineage cocktail, CD80, HLA-DR (APC), CD3

(APC-Cy 7), CD25, IL-10, IL-12 and rat IgG2a were obtained from BD Biosciences, and all other antibodies were purchased from Cambridge Bioscience Ltd., UK.

2.6. Flow cytometric analysis

Samples were analysed using a FACSCanto II flow cytometer (BD, UK). Data were analysed by superenhanced Dmax (SED) normalised subtraction using FlowJo software.

2.7. Statistical analysis

Statistical analysis was performed using Mini Tab 16.0. Data were tested for normality and transformed using the Johnson Transformation where appropriate. Significant differences ($P < 0.05$) were evaluated by the Student's t-test or two-way ANOVA using the General Linear Model, followed by appropriate post-hoc tests with Bonferroni correction. All data are shown as mean $\pm$ SE (standard error).

3. Results

3.1 Ageing impairs cytokine production by DCs, but not surface marker expression

DCs in LDC preparations from human blood were identified as HLA-DR⁺ and negative for a lineage cocktail containing CD3, CD14, CD19 and CD20 antibodies (Figure 2A). The proportion of DCs was similar in the young and older subjects. Cell surface expression of CD80, CD40 and CCR7, but not CD86, were significantly up-regulated after conditioning with LPS (Fig. 3). None of the surface markers, or their up-regulation by LPS, were affected by ageing (Fig. 3).

There were significant effects of ageing on LPS-induced production of TNF- α and IFN- γ by DCs (Fig. 4). LPS induced production of TNF- α and IFN- γ in DCs from the young subjects only; DCs from the older subjects were unresponsive (Fig. 4). It was notable that IFN- γ production by unstimulated DCs from older subjects was higher than that from young

subjects (Fig. 4). LPS induced production of IL-10, IL-12 and TGF- β to a similar degree in young and older DCs data not shown.

3.2. Influence of ageing on DC-induced activation of T cells

LPS/DC-induced T cell proliferation was significantly affected by the age of both the DCs and the T cells (Fig. 5). In MLR, the highest rate of proliferation was observed when DCs from young subjects were co-incubated with T cells from young subjects (Fig. 5). When LPS-stimulated DCs from young subjects were co-incubated with T cells from older subjects, proliferation was significantly reduced (Fig. 5). DCs from older subjects exhibited a poor ability to induce proliferation of T cells, regardless of whether the T cells were donated by young or older subjects (Fig. 5).

Activation of young and old T cells in the MLR was assessed by expression of CD25 on CD4⁺ and CD8⁺ T cells (Fig. 6). Exposure of young T cells to either unstimulated DCs or LPS-stimulated DCs increased expression of CD25, but there was no response of T cells from older subjects; this was the case for both CD4⁺ and CD8⁺ T cell populations (Fig. 6). The effects of ageing were particularly notable for the CD8⁺ T cells, where induction of CD25 expression by young CD8⁺ T cells was significantly greater if the LPS-stimulated DCs were donated by young subjects (Fig. 6). It was also notable that neither young DCs nor older DCs were able to induce CD25 expression on older CD8⁺ T cells, indicating significant unresponsiveness of CD8⁺ T cells from older subjects in the MLR (Fig. 6).

3.3. Influence of ageing on DC-induced homing ability of T cells

Expression of integrin β 7 confers gut-homing ability (Wagner, Lohler et al. 1996; Agace, Higgins et al. 2000). Although there was a greater proportion of integrin β 7⁺ older T cells at baseline, LPS-stimulated DCs upregulated integrin β 7 expression by young T cells, but not by older T cells (Fig. 7), suggesting impaired responsiveness of older T cells to DC priming (Fig. 7).

3.4. Ageing impairs production of IL-12 and TGF- β by T cells in the MLR

Unstimulated T cells from old subjects produced significantly higher levels of IFN- γ than that from young subjects, but production of TNF- α , IL-12, TGF- β and IL-10 by unstimulated T cells was not different between the two age groups (Fig. 8). All of the cytokines, except for IFN- γ , were inducible by DC stimulation; however DCs induced IL-12 and TGF- β production by young T cells only (Fig. 8). In contrast, IL-10 production by old T cells stimulated with DCs was significantly greater than that of young T cells (Fig. 8).

There was little difference in the capacity of young vs old DCs to induce production of cytokines by T cells, with the exception of IL-12 production in DC-stimulated old T cells and IFN- γ expression on young T cells treated with LPS-stimulated DCs being greater when DCs were derived from young subjects (Fig. 8).

4. Discussion

This study demonstrates that cytokine production by DC and the capacity of DCs to trigger T cell responses in the MLR are significantly impaired by ageing. The use of blood-enriched DCs (LDCs) avoids the long culture period and the conditions required for *in vitro* differentiation and the ability of LDCs to participate in MLRs has been demonstrated previously (Knight, Farrant et al. 1986; Holden, Bedford et al. 2008). In the current study, the expression of the co-stimulatory molecules, CD40 and CD80, by unstimulated DCs was comparable in young and old subjects, suggesting that ageing has little influence on the maturation of DCs in healthy older subjects; this is consistent with several other studies (Steger, Maczek et al. 1996; Pashenkov, Kouwenhoven et al. 2000; Agrawal, Agrawal et al. 2007; Della Bella, Bierti et al. 2007; Pereira, Duarte de Souza et al. 2011; Tan, Cavanagh et al. 2012). The response to LPS was also preserved to some extent during ageing, consistent with the studies of Agrawal, Agrawal et al. (2007) and Ciaramella, Spalletta et al. (2011),

who used monocyte-derived DCs. Furthermore, although Della Bella, Bierti et al. (2007) demonstrated greater maturation status of circulating DCs in older subjects, there was a comparable response to LPS of DCs from young and older subjects. Some studies reported reduced expression of co-stimulatory molecules and cytokine production in frail elderly subjects (Uyemura, Castle et al. 2002), suggesting that health status might play a role in age-associated changes in DC function. The ability of DCs to migrate to secondary lymph nodes to elicit appropriate T and B cell mediated responses following the capture of antigen is critical and requires expression of CCR7 (Steger, Maczek et al. 1996; Linton, Li et al. 2005; Toapanta and Ross 2009). The current study suggests that CCR7 expression is preserved during ageing; this was also observed by Agrawal, Agrawal et al. (2007) using monocyte-derived DCs.

Impairment of T cell activation by ageing DCs has been suggested to contribute to immunosenescence (Pereira, Duarte de Souza et al. 2011). In the current study, the greatest rate of T cell proliferation was observed in young T cells stimulated by young LPS-stimulated DCs. Older LPS-stimulated DCs were unable to induce proliferation to the same extent in young T cells. There is little comparative data in the literature, but Agrawal, Agrawal et al. (2007) demonstrated that monocyte-derived DC-induced proliferation of CD4⁺ naïve T cells was impaired when the DCs were derived from older subjects. In contrast, Steger, Maczek et al. (1996) demonstrated no impairment of the ability of human peripheral blood mononuclear cell-derived DCs from older subjects to stimulate T cell lines. The use of cell lines may be a critical methodological difference contributing to the difference in outcome. A novel aspect of the current study was the use of either young or old human T cells in the MLR. In the current study, LPS/DCs induced the proliferation of older T cells to a lower degree than young T cells, even when DCs were derived from young subjects. Such intrinsic defects in responsiveness of aged T cells to antigen have been reported in an

allogeneic MLR model using murine myeloid bone marrow-derived DCs (Tesar, Walker et al. 2006). Overall, this suggests that both DC and T-cell function are affected by ageing.

It is notable that in the current study, activation of CD8⁺ cytotoxic T cells in young subjects by LPS-stimulated DCs was preferentially impaired; CD4⁺ T cells remained relatively unaffected by ageing of DCs. However, Agrawal, Agrawal et al. (2007) demonstrated age-related impairment of proliferation of influenza specific CD4⁺ T cells by monocyte-derived DCs; the reasons for this are unclear, but may be related to differences in cell type and stimulus. In general, ageing results in a greater diminution and dysregulation of circulating CD8⁺ naïve T cells (Ferrando-Martinez, Ruiz-Mateos et al. 2011), greater loss of telomerase activity following pathogen stimulation (Valenzuela and Effros 2002), and greater age-associated changes in TNF- α secretion by CD8⁺ T cells than their CD4⁺ counterparts (Schindowski, Fröhlich et al. 2002). Although the mechanisms underlying the greater impairment of CD8⁺ T cell responsiveness to DCs in the current study are not clear, it is reasonable to suggest that decreased TNF- α production by DCs may be involved (Liu, Nahar et al. 2012).

Pereira, Duarte de Souza et al. (2011) suggested that T cell defects during ageing were related to multiple alterations on aged DCs. In the current study, ageing reduced production of TNF- α and IFN- γ by DCs in response to LPS stimulation. This has potentially important implications, since reduced TNF- α and IFN- γ production by plasmacytoid DCs from older subjects, caused by defects in TLR signalling, is associated with an inability to generate an effective antibody response to influenza vaccination (Panda, Qian et al. 2010). A role for TNF- α in DC-induced T cell proliferation is also evident from clinical data suggesting poor stimulation of T cell activity by DCs from rheumatoid arthritis patients treated with anti-TNF- α antibodies (Baldwin, Ito-Ihara et al. 2010; Liu, Nahar et al. 2012). Thus, impaired

production of TNF- α and IFN- γ by older DCs could result in a weak response to vaccination and contribute to the dysregulation of DC-induced T cell proliferation in elderly subjects. However, the literature is not entirely consistent with respect to the effects of ageing on overall DC cytokine secretion profiles (Lung, Saurwein-Teissl et al. 2000; Wong, Magnusson et al. 2010) (Agrawal, Agrawal et al. 2007; Agrawal, Tay et al. 2009) (Della Bella, Bierti et al. 2007; Panda, Qian et al. 2010; Ciaramella, Spalletta et al. 2011). It is likely that the use of different stimuli (e.g. influenza virus, TLR agonists, self-DNA, LPS), species, methodology (e.g. intracellular vs secreted cytokines) and source of DCs may account for at least some of the inconsistency.

The current study demonstrated that although LPS-stimulated IFN- γ production by older DCs was impaired, basal production of IFN- γ by older DCs was higher than young subjects. An ageing-associated increase in IFN- γ has previously been reported (Sakata-Kaneko, Wakatsuki et al. 2000; Lio, Scola et al. 2002) and is suggested to contribute to the phenomenon of ‘inflamm-ageing’ (Fridman and Tainsky 2008), although there is little data on the influence of ageing on basal IFN- γ production by circulating DCs. The data herein suggests that DCs might contribute to the systemic accumulation of IFN- γ associated with ageing.

Since co-culture of young DCs with young T cells resulted in the greatest rate of proliferation and activation of T cells, it was expected that the worst combination would be old DCs and old T cells. However, this was not the case; young DCs were equally poor at stimulating old T cells as were old DCs, suggesting that intrinsic defects in T cells outweigh the impact of ageing DCs. The literature is not clear on whether impaired T-cell responses during ageing are exclusively due to intrinsic defects or influenced by ageing of DCs. Experiments involving transfer of young/old murine T cells to young/old recipients demonstrated that age-related impairment of T-cell activation was mainly due to intrinsic T-

cell defects and not critically dependent on the ageing of antigen-presenting cells (Tesar, Walker et al. 2006). However, a recent study suggested that reduced function of aged T cells was intimately related with impairment of DC function (Pereira, Duarte de Souza et al. 2011). The current study supports the former view.

The most dramatic effects of ageing on cytokine production by T cells in the MLR were for IL-12, TGF- β and IL-10. IL-12 production by older T cells was significantly lower than that by young T cells, and the capacity of older DCs to induce IL-12 production by older T cells was reduced compared to that of young DCs. As a result, ageing of both DCs and T cells resulted in impaired IL-12 production in the MLR. DC-induced TGF- β production by T cells was significantly impaired by ageing, but the defect appeared to reside primarily within T cells, since the ability of older DCs to induce TGF- β production by young T cells was preserved. In contrast to IL-12 and TGF- β , IL-10 production by older T cells in response to DC stimulation was augmented compared with that by young T cells, regardless of whether the DCs were derived from young or older subjects. It is noteworthy that pre-stimulation of DCs with LPS did not further increase IL-10 production by older T cells, which is suggestive of a defect in TLR-dependent immunity. It has been suggested that high production of IL-10 in the elderly indicates development of immune deficiency (Gregg, Smith et al. 2005) and is associated with a weak response to influenza vaccination (Corsini, Vismara et al. 2006). However, the impact of ageing on T cell immunity is complex and may involve defects in cell activation, co-stimulatory signalling and intracellular signalling pathways. Further studies are required to understand the selective nature of the effects of ageing on DC-induced T cell activation.

The β 7 integrin family plays an important role in the homing of T lymphocytes to the gastrointestinal mucosa, and this study shows, for the first time, that T cells from older

subjects were unresponsive to upregulation of integrin $\beta 7$ by DCs. This has potentially important implications, as it is indicative of reduced T-cell migration to intestinal sites, and therefore mucosal immunity (Gorfu, Rivera-Nieves et al. 2009), and may play a role in the greater susceptibility of older individuals to gut-related infections and gastroenteritis (Gavazzi and Krause 2002; Hébuterne 2003). Interestingly, unstimulated T cells from the older subjects expressed significantly higher levels of integrin $\beta 7$ than the young subjects. It could be speculated that greater trafficking of T cells to the intestine could increase the risk of inflammatory disease in older individuals in normal non-pathogenic conditions (Aranda, Sydora et al. 1997; Souza, Elia et al. 1999; Sydora, Wagner et al. 2002).

5. Conclusions

In summary, DC maturation in response to LPS was maintained to some degree during ageing, but the capacity to induce T-cell activation was significantly impaired. This work highlights the challenges associated with developing therapeutic agents for immunosenescence, the impact of which is clearly complex and far-reaching. Finally, it demonstrates that the age of the DC and T cell donor is a critical factor in determining the outcome of the MLR, and emphasizes the importance of stringent controls for age.

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Figure legends

Fig.1 In vitro study design. DC-enriched low density cells (LDCs) were collected from healthy young and older subjects and cultured in the presence or absence of LPS for 24h. After the incubation, some DCs were used for analysis of DC phenotype and cytokine production, and the remainder were used for DC-priming of T cells. T cells from healthy young or older subjects were co-cultured with young or older DCs for 5 days. The controls were DCs or T cells incubated only with medium.

Fig. 2 Gating strategy for DCs and T cells. (A) LDCs were gated in the FSC/SSC plot (upper). These gated cells were separated according to expression of Lineage cocktail (Lin contains anti-CD3, CD14, CD19 and CD20) and HLA-DR; DCs were positive to HLA-DR but negative to Lin (lower). (B) In the MLR culture, T lymphocytes were identified by anti-CD3 staining.

Fig.3 Effects of ageing on surface marker expression on DCs. Data are mean $\pm$ SE for n=8 samples from each group. Data were normalized by the Johnson Transformation. There was a significant effect of LPS ($P<0.01$) on expression of all surface markers except CD86, but no effect of age (two-way ANOVA). Significant differences are denoted as ^a $P<0.05$, ^b $P<0.01$ relative to control for the same age group (post-hoc tests with Bonferroni correction).

Fig.4 Effects of ageing on intracellular cytokine production by DCs. (A) Representative intracellular cytokine production by DCs after superenhanced Dmax (SED) normalised subtraction. (B) Data are mean $\pm$ SE for n=8 samples from each group. Data were normalized by the Johnson Transformation. There was a significant effect of age ($P<0.05$) on expression of TNF- α and IFN- γ and of LPS ($P<0.001$) on expression of all cytokines (two-way ANOVA). Significant differences are denoted as ^a $P<0.05$, ^b $P<0.01$ relative to control for the same age group (post-hoc tests with Bonferroni correction).

Fig.5 Effects of ageing on LPS/DC-induced proliferation of T cells. (A) Representative flow cytometric analysis of T cell proliferation in the absence of LPS/DCs and LPS/DC-induced proliferation of T cells. (B) % of T cells dividing induced by LPS/DCs. Data are mean $\pm$ SE for n=8 samples from each group. Data were normalized by the Johnson Transformation. There was a significant effect of age ($P<0.05$) and LPS/DC

exposure ($P<0.05$) on T cell proliferation (two-way ANOVA). Significant differences are denoted as ^a $P<0.05$ relative to no DC control for T cell with the same age group (post-hoc tests with Bonferroni correction).

Fig.6 Effects of ageing on DC-induced CD25 expression on T cells. Data are mean $\pm$ SE for $n=8$ samples from each group. Data were normalized by the Johnson Transformation. There was a significant effect of ageing ($P<0.05$) on CD25 expression by $CD8^+$ T cells and of DC exposure ($P<0.05$) on CD25 expression by both $CD4^+$ and $CD8^+$ young T cells (two-way ANOVA). Significant differences are denoted as ^N $P<0.05$ relative to the no-DC controls for T cells within the same age group; ^D $P<0.05$ relative to DC-stimulated T cells (without LPS) within the same age group (post-hoc tests with Bonferroni correction).

Fig.7 Effects of ageing on DC-induced expression of integrin $\beta 7$ on T cells. Data are mean $\pm$ SE for $n=8$ samples from each group. Data were normalized by the Johnson Transformation. There was a significant effect of age ($P<0.05$) and treatment ($P<0.05$) on expression of integrin $\beta 7$ (two-way ANOVA). Significant differences are denoted as ^N $P<0.05$ relative to the no-DC control for T cells within the same age group; ^D $P<0.05$ relative to DC-stimulated T cells (without LPS) within the same age group (post-hoc tests with Bonferroni correction).

Fig.8 Effects of ageing on DC-induced intracellular cytokine production by T cells. Data are mean $\pm$ SE for $n=8$ samples from each group. Intracellular cytokine production was assessed, as described in Materials and Methods, within the $CD3^+$ T cell population. Data were normalized by the Johnson Transformation. There was a significant effect of age ($P<0.05$) on expression of IL-12, IL-10, TGF- β and IFN- γ and treatment ($P<0.05$) on all cytokines except IFN- γ on Young and Old T cells, and IL-12 on Young T cells (two-way ANOVA). Significant differences are denoted as ^N $P<0.05$ relative to the no-DC control for T cells within the same age group; ^D $P<0.05$ relative to DC-stimulated T cells (without LPS) within the same age group; ^T $P<0.05$ relative to T cells from older subjects for the same condition (post-hoc tests with Bonferroni correction).