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Comprehensive analysis of MHC-II expression in healthy human skin

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Abstract

A number of antigen presenting cells (APC) expressing MHC class II have been identified in healthy human skin including the Langerhans cells of the epidermis and the three recently defined dermal APC subsets. It is well documented that in other tissues HLA-DR expression is not exclusive to APCs. Following a comprehensive analysis of the cells in human skin using flow cytometry and fluorescence immunohistochemistry we have identified additional cell subsets that express HLA-DR. Using markers exclusive for blood and lymphatic endothelium we demonstrated that both of these cell populations have the capacity to express HLA-DR. In addition a small subset of dermal T-lymphocytes were found to express low-level HLA-DR suggesting an activated phenotype. Dermal T-lymphocytes were often in intimate contact with either $CD1a^+$ $CD207^-$ dermal APCs or $CD1a^+$ $CD207^+$ dermal Langerhans cells, possibly explaining the activated phenotype of a subset of dermal T-lymphocytes.

Keywords

Antigen-Presenting Cells; Human; Major Histocompatibility Complex; Skin; T-lymphocytes

Introduction

Major histocompatibility complex class I and II molecules play a vital role in initiating immune responses by presenting peptide antigens to CD8 and CD4 T-lymphocytes respectively. Early research indicated that expression of MHC class II (MHC-II) was restricted to particular APC subsets including professional APCs or dendritic cells, monocytes, macrophages and B-lymphocytes. More recent research however has shown that MHC-II is not exclusively expressed by these populations and can be found on non-APC subsets, such as activated T-lymphocytes (1) and basal vascular endothelial cells (2, 3). Although a substantial amount of research assessing the functional relevance of MHC-II expression on non-APCs has been conducted its precise role on these cells remains unresolved. It has been postulated that endothelial cells may have the capacity to acquire, process and present antigen to memory T-lymphocytes (3) whilst T-lymphocytes which express MHC-II and can also provide co-stimulatory signals (4-6) might have the potential to mediate immune responses (7).

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In the skin a number of APC subsets expressing MHC-II have been identified, including the well characterised Langerhans cells of the epidermis (8, 9) and the dermal APCs (10-13). We recently reported that three APC subsets exist in the healthy human dermis, $CD1a^+$ and $CD14^+$ dermal APC and migratory Langerhans cells (13). Our data suggested that $CD1a^+$ dermal APC are likely to represent the dermal APC population capable of migration to the lymph nodes and stimulating naïve T-lymphocytes (13). In contrast the less mature $CD14^+$ dermal APC were unable to migrate in response to lymph node homing chemokines (13). This $CD14^+$ subset may represent a cutaneous macrophage population or a precursor for other APC subsets in the skin as previously suggested (12), however the exact function of these cells remains unresolved. In addition to the epidermal Langerhans cells, we detected a number of $CD207^+$ migratory Langerhans cells in the dermal papillae region (13), consistent with the Langerhans cells' ability to migrate from the epidermis, via the dermis to the afferent lymphatic vessels and subsequently the draining lymph nodes where they can initiate an immune response (14-16). In addition to these APC populations healthy human skin may contain non-APCs that express MHC-II similar to those detected in other tissues. If $MHC\text{-}II^+$ non-APC subsets do exist in the healthy human skin, these cells may have the potential to present peptide antigen and subsequently influence the cutaneous immune environment. We therefore conducted a comprehensive study using flow cytometry of single cell suspensions from the epidermis and dermis, and multi-colour immunohistochemical analysis to identify the cells in healthy human skin that express MHC-II. We show that MHC-II expression is not restricted to the recognised cutaneous APC subsets, and that non-APC populations account for a substantial proportion of the MHC-II positive cells in human dermis.

Results

Is MHC-II expression restricted to the recognised cutaneous APC subsets?

We used a rapid enzymatic digestion protocol for the dissociation of human skin into single cell suspensions from dermis and epidermis (13). The HLA-DR positive cells in the dermal and epidermal cell suspensions were then detected using flow cytometry.

As we recently reported, three distinct APC subsets could be identified in cell suspensions from healthy human dermis: $CD14^+$ dermal APC; $CD1a^+$ dermal APC; and migratory Langerhans cells (13). $CD14^+$ dermal APC did not express surface CD1a and could readily be detected using a CD14 antibody. All $CD14^+$ dermal APC expressed a moderate to high level of HLA-DR (Fig 1*a*). As we previously reported (13) the $CD1a^+$ cells detected in the dermis split into two distinct populations, $CD1a^{+mid}$ cells which were $CD207^-$ (Fig 1*c*) and represent the $CD1a^+$ dermal APC subset, and $CD1a^{+high}$ cells which expressed CD207 (Fig 1*c*) and are likely to be Langerhans cells migrating from the epidermis to the afferent lymphatic vessels. All of the $CD1a^{+mid}$ dermal APC expressed moderate to high levels of HLA-DR (Fig 1*b*), however the level of expression was marginally higher than that expressed by $CD14^+$ dermal APC (Fig 1*a*). The $CD1a^{+high}$ migratory Langerhans cells expressed a slightly higher level of HLA-DR when compared with $CD1a^{+mid}$ dermal APC (Fig 1*b*). We were unable to detect $BDCA\text{-}2^+$ $HLA\text{-}DR^+$ plasmacytoid dendritic cells in the dermis (data not shown).

In cell suspensions from the epidermis two distinct $CD1a^+$ APC populations were present. The dominant subset expressed a high level of CD1a and CD207 (Fig 1*e*) typical of epidermal Langerhans cells (17). The majority of these epidermal Langerhans cells expressed a similar level of HLA-DR (Fig 1*d*) when compared with the dermal APC populations (Fig 1*a-b*), however a minor Langerhans cell population expressing a higher level of HLA-DR was also detected (Fig 1*d*). The second $HLA\text{-}DR^+$ subset expressed a moderate level of CD1a and was devoid of surface CD207 (Fig 1*e*); these cells are likely to

represent contaminating CD1a^{+mid} dermal APC (Fig 1*a-b*) as no CD1a^{+} CD207^{-} cells were detected in the epidermis using immunohistochemistry (data not shown).

Immunohistochemistry was used to confirm HLA-DR expression by each of the cutaneous APC subsets *in situ*. CD14^{+} dermal APC were distributed throughout the dermis consistent with earlier data (13) and expressed HLA-DR, however the cells located in the more superficial areas just below the epidermis appeared to express a slightly higher level of HLA-DR when compared with those in the deeper regions of the dermis (Fig 1*f*). A proportion of the CD14^{+} dermal APC were found associating with vascular like cellular clumps in the dermal papillae (Fig 1*f*). Background staining was observed in the epidermis in Fig 1, however as CD14^{+} cells are not present in the epidermis (13) this did not interfere with image interpretation. CD1a^{+} APC were primarily located in the dermal papillae, often associating with cellular clumps (Fig 1*g*) previously identified as lymphatic endothelium (13). As all CD1a^{+} APC in the dermis expressed HLA-DR (Fig 1*g*), we can conclude that CD1a dermal APC express this molecule *in situ*. Langerhans cells expressing CD1a and CD207 were equidistantly spaced throughout the epidermis consistent with earlier reports (18, 19) and expressed HLA-DR *in situ* (Fig 1*h*). However staining of skin sections with anti-HLA-DR alone revealed that HLA-DR expression by epidermal Langerhans cells was weak and substantially lower than that expressed by cells in the dermis (data not shown). This was inconsistent with the flow cytometry data that showed Langerhans cells and dermal populations expressed a comparable moderate to high level of HLA-DR (Fig1 *a, b* and *d*). It therefore appears that the Langerhans cells up-regulated HLA-DR during the cell preparation procedure, as previously reported (20). CD207 staining of cells in the dermis provided confirmation that migratory Langerhans cell expressed HLA-DR (Fig 1*h*).

The flow cytometry data presented in Figure 1 *d* and *e* showed that most of the cells expressing HLA-DR in the epidermal cell preparations expressed CD1a; hence the majority of HLA-DR^{+} cells in the epidermis were APC. In contrast the dermis contained a substantial proportion of HLA-DR^{+} cells that were not positive for CD14 or CD1a (Fig 1*a-b*) indicating they do not belong to the recognised dermal APC subsets. This observation was validated using flow cytometry (Fig 1*i*) and immunohistochemistry (Fig 1*j*). Both sets of data clearly show that when antibodies recognising CD14 and CD1a are used simultaneously, they do not label all of the HLA-DR^{+} cells present in the dermis. The level of HLA-DR expressed by the non-APC subsets was lower than that expressed by the dermal APC populations (Fig 1*i*). A large proportion of the HLA-DR^{+} non-APC subsets was located in cellular clumps in the dermal papillae, whilst the remainder of these cells were scattered throughout the upper region of the dermal papillae just below the epidermis (Fig 1*j*).

Identifying the non-APC populations that express MHC-II in the healthy human dermis

Additional flow cytometry experiments were conducted to identify the HLA-DR^{+} populations that were not recognised using the markers for cutaneous APCs. Figure 2 shows that dermal cell suspensions contained a large number of CD3^{+} T-lymphocytes (*a*) including CD4^{+} T-lymphocytes (data not shown) and endothelial cells from blood vessels (*c*) (identified by expression of CD144/VE-cadherin (21)) and lymphatic vessels (*d*) (identified by expression of podoplanin (22, 23)). Very few CD19^{+} B lymphocytes were detected in the dermal cell preparations (*b*). A small proportion of the T-lymphocytes were found to express low-level HLA-DR (Fig 2*a*), much lower than that expressed by the dermal APCs (Fig 1*a, b* and *d*). The majority of the CD144^{+} blood endothelial cells expressed moderate to high levels of surface HLA-DR (Fig 2*c*) similar to that expressed by the dermal APC subsets. In contrast only a minor proportion of the lymphatic endothelial cells expressed surface HLA-DR (Fig 2*d*) again at a comparable level to dermal APCs.

Immunohistochemistry experiments were conducted to confirm the expression of HLA-DR by T-lymphocytes and blood and lymphatic endothelial cells *in situ*. Figure 2*e* shows T-lymphocytes some of which express HLA-DR, in close proximity to two capillary like structures in the dermis which also express HLA-DR. HLA-DR$^+$ T-lymphocytes were often located close to endothelial like cellular clumps, however they were also occasionally solitary. The CD144$^+$ blood endothelial cells in figures 2*f-g* appear as small capillaries, although CD144$^+$ endothelial cells also form larger vessels deep in the dermis (data not shown). The CD144$^+$ blood endothelial cells exhibited weak punctate HLA-DR staining consistent with endosomal HLA-DR; the expected strong surface expression detected using flow cytometry was not evident. It is therefore possible that surface HLA-DR expression was up regulated during the tissue digestion procedure. The strong HLA-DR expression detected on a population of CD144 negative cells surrounding the CD144$^+$ capillaries (Fig 2*f-g*) is likely to be on APCs, as shown in Fig 1.

The distribution and cellular structure of the lymphatic endothelium was strikingly different from that of blood endothelium; lymphatic endothelial vessels appeared as cellular clumps and were concentrated in the dermal papilla (Fig 2*h*). In figure 2*h* a subset of lymphatic endothelial cells making up one of these clumps expressed HLA-DR *in situ*. Surrounding the podoplanin$^+$ lymphatic endothelial cells there were podoplanin negative HLA-DR$^+$ cells (Fig 2*h*), these are likely to be cutaneous APCs or possibly HLA-DR$^+$ T-lymphocytes draining into afferent lymphatic vessels.

Flow cytometry data obtained using antibodies recognizing each of the cutaneous APC subsets, B and T-lymphocytes and blood and lymphatic endothelial cells indicated that practically all HLA-DR$^+$ cells in the healthy human dermis were accounted for using these cellular markers (Figure 2*i*).

Dermal APCs form close associations with dermal T-lymphocytes

As a substantial number of T-lymphocytes were detected in the dermis (Fig 2*a* and *c*), we wanted to establish whether these cells formed associations with the cutaneous APCs *in situ.*

Initially flow cytometry was used to assess whether T-lymphocytes associated with APCs in the dermis. Populations of CD1a^{high} CD3$^-$ dermal Langerhans cells, CD1a^{mid} CD3$^-$ dermal APCs and CD3$^+$ CD1a$^-$ T-lymphocytes were present in the dermal cell preparations (Fig 3*a*). Two additional minor populations were also detected; these were CD1a^{high} CD3$^+$ and CD1a^{mid} CD3$^+$ (Fig 3*a*). The forward and side scatter profiles of each of these populations were plotted; the CD1a^{high} CD3$^+$ (Fig 3*e*) and CD1a^{mid} CD3$^+$ (Fig 3*f*) subsets were both larger and slightly more granular than their CD1a^{high} CD3$^-$ (Fig 3*b*) and CD1a^{mid} CD3$^-$ (Fig 3*c*) counterparts. Cumulatively the co-staining of markers specific for each cell type, and the shifts in the forward and side scatter profiles indicates that these minor populations are T-lymphocytes aggregated with CD1a^{high} dermal Langerhans cells and CD1a^{mid} dermal APCs. CD14 dermal APCs were rarely associated with T-lymphocytes (Fig 3*g*).

Immunohistochemistry was used to confirm that both CD1a$^+$ dermal APCs and dermal Langerhans cell were in intimate contact with T-lymphocytes *in situ.* Figure 3*h-j* clearly illustrates that both CD1a$^+$ CD207$^+$ dermal Langerhans cells and CD1a$^+$ CD207$^-$ dermal APCs formed close associations with CD3$^+$ dermal T-lymphocytes *in situ*. The site of contact between the dermal APC and the T-lymphocytes often appeared yellow indicating co-staining of the cellular markers, confirming the intimate nature of these cellular interactions. Furthermore, dendrites were often observed extending out into the dermis, on occasion reaching out to, and encapsulating T-lymphocytes (Fig 3*i-j*). The T-lymphocyte and APC aggregates were often located within cellular clumps in the dermal papillae likely to be draining lymphatic vessels however solitary T-lymphocyte and APC aggregates were

also observed away from vascular areas (Fig 3 *h* and *j*). The T-lymphocyte and APC aggregates were rare; approximately three $CD1a^+$ dermal APC: T-lymphocyte aggregates were detected per $50mm^2$, whilst only one or two dermal Langerhans cell: T-lymphocyte aggregates were detected per $50mm^2$ skin section.

Assessing the activation status of T-lymphocytes in the dermis

The flow cytometry data shown in Figure 4 shows that a proportion of dermal T-lymphocytes expressed low-level surface CD25 (*a*), however no CD38 (*b*) or CD154 (*c*) positive cells were detected. A population of $CD3^-$ $CD25^+$ cells was also detected in the dermis (Fig 4a), and is likely to be CD1a dermal APCs and migratory Langerhans as these were the only additional $CD25^+$ cells detected in the dermis (data not shown).

Discussion

In this study we demonstrate that the recognised APC populations $CD14^+$ dermal APC, $CD1a^+$ dermal APC and migratory Langerhans cells are not the only cells in the healthy human dermis that express the peptide presentation molecule MHC-II. Although HLA-DR expression by cutaneous endothelium has been described previously (24), for the first time we use antibodies exclusive for blood and lymphatic vessels to show that cells from both of these endothelial subsets can express MHC-II in human skin. Hence both blood and lymphatic endothelial cells in the dermis may have the potential to present peptide antigen and subsequently be involved in modulating the cutaneous immune environment, similar to endothelium in other tissues (3). We also detected a minor MHC-II positive T-lymphocyte subset indicating that these cells have a activated phenotype (1). The presence of activated T-lymphocytes in the healthy human skin was unexpected, however their close association with two of the dermal APC populations may explain their activation status. Although T-lymphocytes have been detected in close opposition with APCs in the healthy and inflamed human dermis before, the precise identity of the APCs involved in these interactions has never been determined (25-27). Using the cutaneous APC distinguishing surface markers we recently defined (13) we demonstrate that both $CD1a^+$ dermal APC and migratory dermal Langerhans cells are in intimate contact with dermal T-lymphocytes, whilst $CD14^+$ dermal APC are not.

As expected, high levels of HLA-DR expression were detected by both flow cytometry and immunohistochemistry on each of the recently defined $CD1a^+$ dermal APCs and dermal Langerhans cells capable of migrating in response to lymph node homing chemokines (13). A high level of surface HLA-DR was also detected on $CD14^+$ dermal APC using flow cytometry, however the immunohistochemistry data illustrated that $CD14^+$ dermal APCs located in the deep dermis expressed lower levels of HLA-DR when compared with the more superficial $CD14^+$ dermal APC just below the epidermis. This discrepancy between techniques may be due to the HLA-$DR^{-/low}$ $CD14^+$ dermal APC undergoing maturation during the tissue dissociation procedure, which is consistent with the current opinion that any kind of skin manipulation will result in the maturation of at least a proportion of cutaneous APCs (9). The reason for the variation in HLA-DR expression by $CD14^+$ dermal APCs *in situ* is uncertain, although there does appear to be a correlation between the level of HLA-DR expression relative to the dermal depth of the cell. It is also interesting that the $CD14^+$ dermal APCs expressing higher levels of HLA-DR were located together with the more mature $CD1a^+$ dermal APC in the upper regions of the dermis, where the lymphatic vessels are concentrated. Epidermal Langerhans cells expressed low-level HLA-DR *in situ*; however following tissue digestion the level of surface HLA-DR detected using flow cytometry was extremely high. Therefore the tissue dissociation procedure may have stimulated the up-regulation of surface HLA-DR by the Langerhans cells in addition to the $CD14^+$ dermal APC. The different level of HLA-DR expression by Langerhans cells and

CD1a$^+$ dermal APC *in situ* supports our earlier observations that these cells represent two distinct populations possibly with different functions. We were unable to detect BDCA-2$^+$ HLA-DR$^+$ plasmacytoid dendritic cells, consistent with the viewpoint that these cells are very rare in healthy skin and only infiltrate the skin in high numbers following inflammation (8).

Once the recognised cutaneous APC phenotypes were accounted for, we discovered that a large proportion of non-APCs also expressed moderate to high levels of HLA-DR. Further investigation revealed that these HLA-DR positive populations were T-lymphocytes and blood and lymphatic endothelial cells. Hence MHC-II is not exclusively expressed by APC subsets in the cutaneous environment.

The small subpopulation of HLA-DR positive T-lymphocytes detected in the dermis may represent an activated subset, as human T-lymphocytes are known to express HLA-DR following stimulation via their T-cell receptor (1). CD25, the high affinity component of the IL2 receptor was also detected on a subset of T-lymphocytes in the dermis, consistent with recent activation. However the activation markers CD38 and CD154 were not expressed on T-lymphocytes extracted from normal dermis, and although it is possible these molecules were cleaved by the enzymes used in preparing our single cell suspensions, such enzyme sensitivity has not previously been reported for these markers. Interestingly the expression of surface CD25 and the absence of the classical activation markers raises the possibility that these cells may be regulatory T-lymphocytes. Although we have not conducted the necessary experiments to confirm a regulatory phenotype a recent publication has shown that CD4$^+$CD25$^+$CD69$^-$ regulatory T-lymphocytes exist in the skin and these cells when enriched suppressed T-lymphocyte proliferation typical of regulatory T-lymphocytes (28).

Although MHC-II expression is often used as a marker of activated human T-lymphocytes, the role of this antigen presentation molecule on T-lymphocytes *in vivo* remains unresolved. It has been proposed that MHC-II$^+$ T-lymphocytes have a role in maintaining immune tolerance (29-31), however it must be recognised that the precise function of MHC-II expressed by T-lymphocytes remains controversial (7).

A number of T-lymphocytes were detected aggregating with CD1a$^+$ dermal APC and migratory Langerhans cells in the dermal single cell suspensions. A similar phenomena has been observed in a previous study where HLA-DR^{high} cells that spontaneously migrated from healthy human skin explants aggregated with memory T-lymphocytes, however the APCs involved in these interactions were not identified (25). This publication also noted that when these aggregates were removed from the cutaneous cell suspensions additional aggregates formed during culture (25). To rule out this possibility, we used immunohistochemistry to confirm that CD1a$^+$ dermal APC and migratory dermal Langerhans cells were engaging with T-lymphocytes *in situ*. An earlier immunohistochemistry study also noted that dermal APCs and T-lymphocytes were often in close apposition in healthy human dermis (26), however, this study did not identify the precise APC subsets associating with the T-lymphocytes either (26). Furthermore, the APC and T-lymphocyte clusters observed were in cellular clumps in the dermal papillae, which we have now identified as lymphatic endothelium, suggesting the populations observed in this earlier study may simply have been draining into the same afferent lymphatic vessel rather than been intimately apposed. For the first time using recently defined cutaneous APC distinguishing surface markers (13) we clearly illustrate that CD1a$^+$ dermal APC and migratory Langerhans cells are the APCs in intimate contact with T-lymphocytes in the healthy human dermis. Furthermore these cutaneous APC and T-lymphocyte aggregates were not only detected in proximity to lymphatic vessels but also away from vascular structures in the healthy human dermis, indicating that these cells were genuinely in contact

with one another rather than just draining into the same afferent lymphatic vessel. Interestingly we did not detect the less mature $CD14^+$ dermal APC subset (13) associating with T-lymphocytes in the dermal cell suspensions or *in situ* using immunohistochemistry. It therefore appears that only migratory Langerhans cells and $CD1a^+$ dermal APC both of which have the capacity to migrate in response to lymph node homing chemokines (13) are able to aggregate with T-lymphocytes *in situ*. Perhaps these two APC subsets are able to initiate a local recall response *in situ* before they migrate to the lymph node as previously suggested (25). Our study was conducted using healthy human skin, interestingly a study assessing inflamed skin has also observed $CD1a^+$ APCs aggregating with $CD4^+$ T-lymphocytes (27). However again many of these aggregates were associated with the cellular clumps we identified as lymphatic vessels and all of the $CD1a^+$ APCs were assumed to be Langerhans cells (27). It will now be interesting to use the distinguishing APC markers we have identified and determine the identity of the APC associating with T-lymphocytes in inflamed skin.

For technical reasons we were unable to determine whether the T-lymphocytes associating with the migratory Langerhans cells and $CD1a^+$ dermal APC were either the HLA-DR or CD25 positive T-lymphocytes observed using flow cytometry. The contiguous nature of the interactions between APC dendrites and the T-lymphocytes (Fig 3 *h-i*) made it impossible to resolve whether co-staining for CD3 and HLA-DR was due to T-lymphocyte expression of HLA-DR or close contact between T-lymphocyte and APC dendrites. CD25 is also expressed by dermal APC, so confirming expression of CD25 by T-lymphocytes in intimate contact with dermal APC faced similar technical difficulty. So although we have provided evidence that $CD1a^+$ dermal APCs and migratory Langerhans cells form close associations with T-lymphocytes in the dermis we are unable to confirm whether these APC subsets have the capacity to activate these T-lymphocytes *in situ*. Additional studies using alternative T-lymphocyte activation markers are required to address these issues.

A large proportion of the blood endothelial cells were shown to express moderate to high levels of surface HLA-DR using flow cytometry. However *in situ* HLA-DR was primarily detected in intracellular compartments in the blood endothelial cells. It is possible that the tissue digestion procedure stimulated relocation of intracellular HLA-DR to the cell surface. Some previous research shows that human blood endothelial cells can basally express MHC-II (3) whilst other work suggests that a cytokine stimulus such as IFNγ is required for its up-regulation (32). It therefore appears that expression of MHC-II by blood endothelium is unique to each tissue and the status of that tissue. The functional role of MHC-II on endothelial cells is unclear, although it has been proposed that endothelial cells have the ability to present microbial antigen to circulating memory T-lymphocytes via MHC molecules and once activated these T-lymphocytes provide soluble and contact dependant signals which influence endothelial cell function, including the regulation of leukocyte recruitment (3). As both blood and lymphatic endothelium in the skin express MHC-II *in situ*, and are capable of moderate levels of cell surface expression, it is possible that cutaneous endothelial cells are involved in stimulating T-lymphocytes in human skin.

Cumulatively these data demonstrate that sub-populations of blood endothelial cells, lymphatic endothelial cells and T-lymphocytes express MHC-II in the healthy human skin. Consequently these cells may have the capacity to present peptide antigen and be involved in modulating the cutaneous immune environment, however the precise mechanisms require further study. The presence of activated T-lymphocytes in the healthy human skin was surprising, however their intimate association with $CD1a^+$ dermal APC and migratory dermal Langerhans cells may explain their activation status.

Methods

Preparation of dermal and epidermal cell suspensions

Fresh skin samples were obtained from healthy patients undergoing breast reduction surgery. Patients gave written informed consent, under a protocol approved by the Auckland Ethics Committee and the Clinical Board of the Counties-Manukau District Health Board. Samples were refrigerated and processed no longer than four hours post surgery.

Subcutaneous tissue was excised and discarded. The trimmed skin was washed with RPMI 1640 (Gibco-BRL, Carlsbad, CA) supplemented with 10% foetal bovine serum (RF10) and digested with 1mg/ml collagenase (Type I) (Gibco-BRL) and 1mg/ml dispase (Gibco-BRL) in RF10 for 2 hours with gentle stirring, at 37°C. The epidermis was peeled off the dermis using forceps. The dermis and epidermis were incubated separately at 37°C for a further 16 hours in RF10 alone, before mechanical disruption by pipetting and filtration through 70μm cell strainers (BD Biosciences, San Diego, CA) to obtain single cell suspensions. Single cell preparations were cryopreserved in 50% RPMI 1640, 40% FBS and 10% DMSO. Cryopreservation and subsequent thawing did not influence cell surface phenotype by flow cytometry when compared with fresh cells (data not shown).

Flow cytometric analysis

Cells suspensions were probed with the following mouse monoclonal antibodies on ice for 45 minutes: podoplanin [clone 18H5] (Abcam, Cambridge, UK); CD207 [DCGM4] (Beckman Coulter, Miami, FL); CD1a [HI149], CD3 [UCHT1], CD25 [M-A251], CD144 [55-7Hh1], CD154 [MK13A4] and HLA-DR [L243] (BD Biosciences, San Diego, CA); CD1a [NA1/34-HLK], CD3 [UCHT1], CD14 [UCHM1] and CD38 [AT13/5] (Serotec, Raleigh, NC). Unconjugated primary antibodies were detected using the corresponding isotype specific goat anti-mouse secondary antibody conjugated to FITC (Southern Biotech, Birmingham, AL) or tagged using Zenon Alexa 488 (Molecular Probes, Eugene, OR). The nuclear stain 7AAD (BD Biosciences, San Jose, CA) was included with each stain, and $7AAD^+$ cells gated out of all analyses to exclude non-viable cells. Stained cells were analyzed immediately using a 4-colour FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Three colour immunofluorescence staining

Fresh skin was embedded in TissueTek OCT compound (Sakura Finetek, CA), snap frozen in liquid nitrogen and sectioned using a cryostat. Sections 5μm thick were fixed with ice-cold acetone and blocked with serum free protein block (DAKO, Glostrup, Denmark). Fixed sections were probed with the following mouse monoclonal antibodies and/or a rabbit polyclonal antibody: podoplanin [clone18H5] (Abcam); CD207 [DCGM4] (Beckman Coulter); HLA-DR [TU36] (Caltag, Burlingame, CA); CD3 [UCHT1] and CD144 [55-7Hh1] (BD Biosciences); CD1a [NA1/34] and CD14 [MEM18] (Serotec); CD3 [rabbit polyclonal] (Zymed, San Francisco, CA). The primary antibodies were detected with the corresponding isotype specific goat anti-mouse or goat anti-rabbit IgG (Southern Biotech and Invitrogen) secondary antibodies conjugated to a fluorochrome (FITC, TRITC or Alexa Fluor 350). The specificity of each secondary antibody was confirmed using a species or isotype mismatched primary antibody.

The slides were mounted using Vector-Shield containing DAPI (Vector, Burlingame, CA). Sections were visualised with a Leica DMRE Fluorescent microscope equipped with the following epi fluorescent filters: UV, 470-490μm and 515-560μm (Leica Microsystems, Heerbrugg, Switzerland). Images were obtained using a Leica DC500 Digital camera and processed using Photoshop (Adobe, San Jose, CA).

Background staining of keratin in the stratum corneum was observed with some antibodies, this has also been encountered in previous studies (10, 33).

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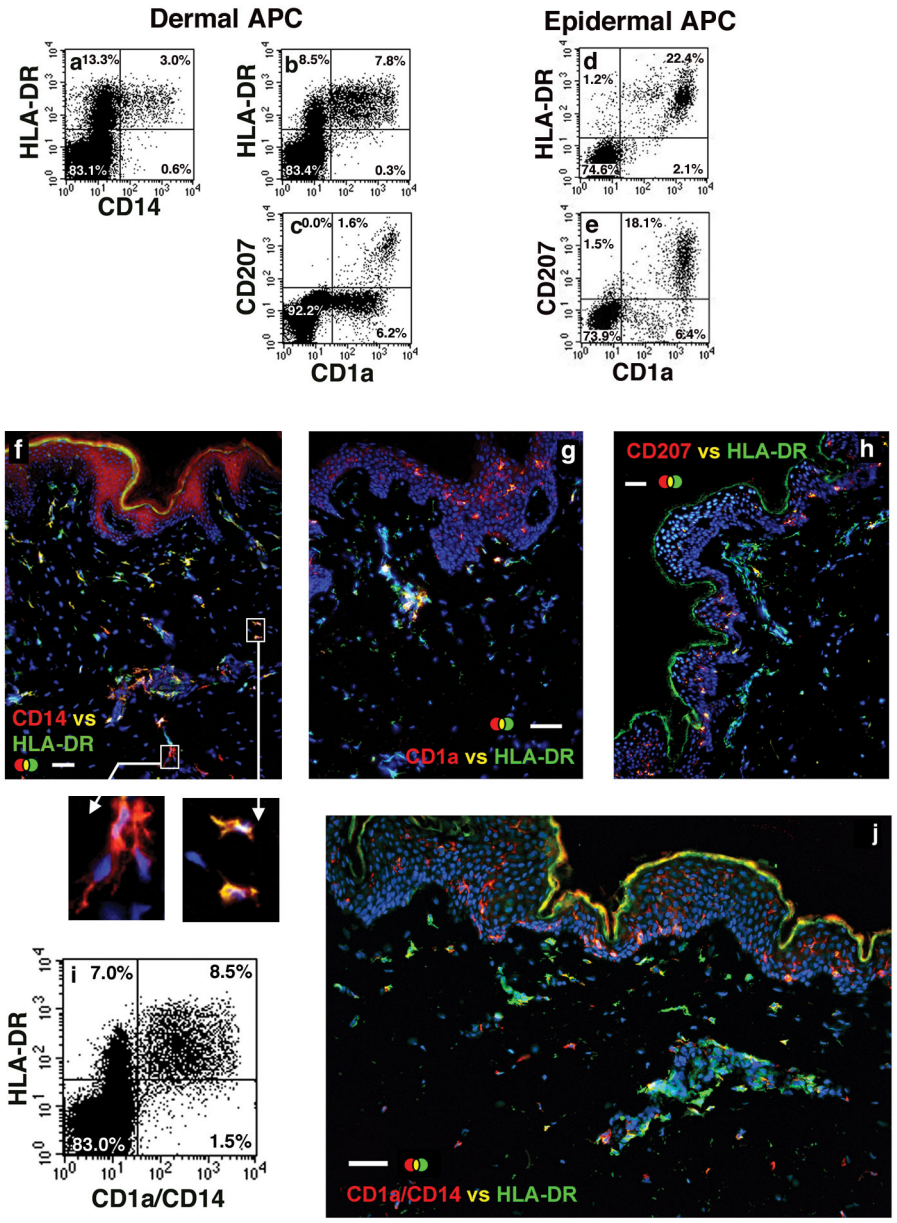

Figure 1. MHC-II is not exclusively expressed by the recognised cutaneous APC subsets in the human dermis

Single cell suspensions from human dermis and epidermis were analyzed using flow cytometry. In the dermal preparations $CD14^+$ dermal APCs (*a*), $CD1a^+CD207^-$ dermal APCs and $CD1a^{high}CD207^+$ dermal Langerhans cells (*b* and *c*) were detected, and each of these APC subsets expressed HLA-DR. In the epidermal preparations $CD1a^+CD207^-$ APCs and $CD1a^{high}CD207^+$ Langerhans cells were detected, again each of these APC subsets expressed HLA-DR (*d* and *e*). All viable $7AAD^-$ cells are shown in these plots. Two-colour fluorescence immunohistochemistry was used to confirm HLA-DR expression by the cutaneous APC subsets *in situ*. $CD14^+$ dermal APCs were distributed throughout the dermis and the majority expressed HLA-DR (*f*). $CD1a^+$ APCs were located in the papillary area of the dermis and the epidermis, both expressed HLA-DR (*g*). $CD207^+$ epidermal and dermal Langerhans cells expressed HLA-DR (*h*). Epidermis is orientated upwards and blue

represents DAPI staining of cell nuclei. Scale bars represent 50μm. Data are representative of three independent experiments.

Single cell suspensions from human dermis were analyzed using flow cytometry to show that cumulatively $CD14^+$ dermal APCs, $CD1a^+$ dermal APCs and $CD1a^+$ dermal Langerhans cells do not account for all of the HLA-DR^+ cells in the healthy human dermis (*i*). All viable $7AAD^-$ cells are shown in this plot. Three-color fluorescence immunohistochemistry also illustrated that HLA-DR expression was not restricted to the recognised cutaneous APC subsets *in situ* (*j*). Epidermis is orientated upwards and blue represents DAPI staining of cell nuclei. Scale bars represents 50μm. Data are representative of three independent experiments.

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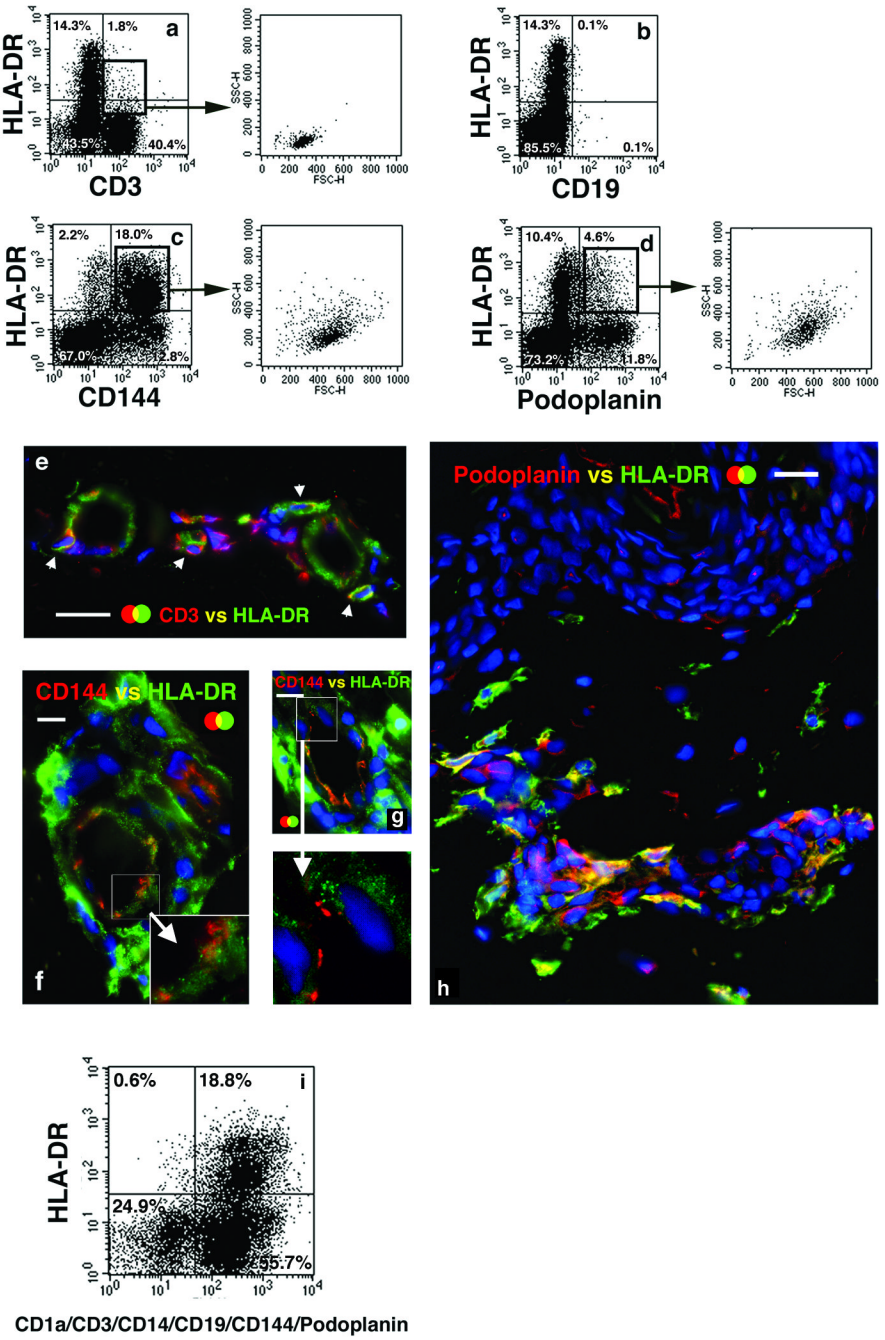

Figure 2. Identifying the non-APC subsets that express MHC class II in the human dermis and do these non-APCs in conjunction with the recognised cutaneous APC subsets account for all of the MHC-II positive cells in the human dermis?

Single cell suspensions from human dermis were analyzed using flow cytometry. A small proportion of the T-lymphocytes in the dermis expressed low level HLA-DR (*a*). In addition the majority of CD144^{+} blood endothelial cells (*c*) and a minor proportion of podoplanin^{+} lymphatic endothelial cells (*d*) expressed moderate to high levels of HLA-DR. The forward and side scatter profile of each of the subsets, are also shown. All viable 7AAD^{-} cells are shown in these plots.

Two-colour fluorescence immunohistochemistry was used to confirm HLA-DR expression by the non-APC subsets *in situ*. $CD3^+$ T-lymphocytes were often located close to dermal vessels and a proportion of these cells expressed HLA-DR (white arrow-head) (*e*). CD144 expression is restricted to the luminal surface of blood endothelial cells. $CD144^+$ blood endothelial cells expressed low-level punctate HLA-DR (*f-g*); $CD144^-$ cells expressing high levels of HLA-DR were often distributed around $CD144^+$ capillaries (*f-g*). A few $podoplanin^+$ lymphatic endothelial cells expressed HLA-DR *in situ* (*h*). Epidermis is orientated upwards and blue represents DAPI staining of cell nuclei. Scale bars represent 25μm (*e*), 10μm (*f-g*) and 20μm (*h*). Data are representative of three independent experiments.

Flow cytometry data obtained using antibodies recognizing each of the cutaneous APC subsets, B and T-lymphocytes and blood and lymphatic endothelial cells confirms that nearly all $HLA\text{-}DR^+$ cells in the healthy human dermis are accounted for using these cellular markers (*i*). All viable $7AAD^-$ cells are shown in this plot.

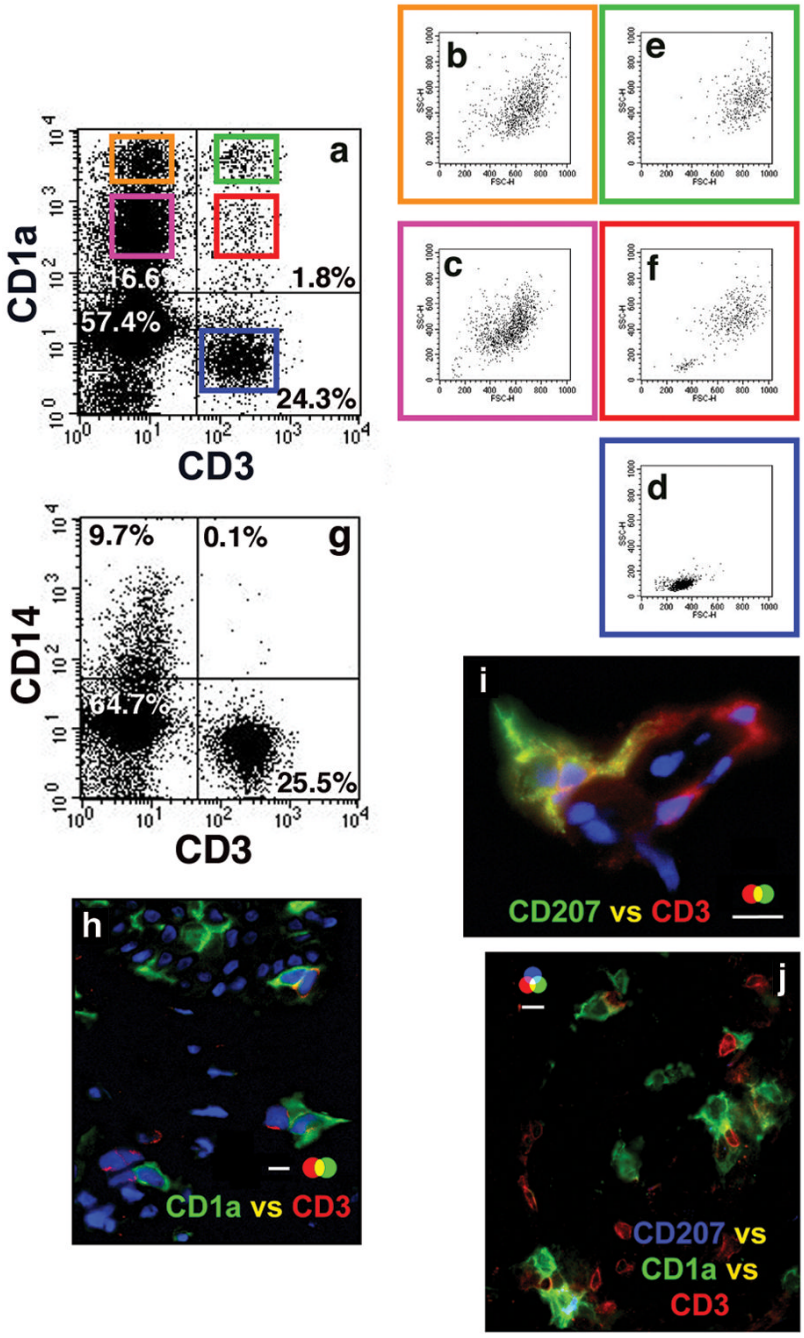

Figure 3. Dermal APCs form close associations with dermal T-lymphocytes

Single cell suspensions from human dermis were analyzed using flow cytometry. Aggregates of CD1a^{mid} dermal APCs and CD1a^{high} dermal Langerhans cells with T-lymphocytes were detected both by co-staining of markers specific for each cell type (*a*), and by shifts in the forward and side scatter profiles (*e-f*) compared with the single cell populations (*b-d*). CD14^{+} dermal APC were rarely detected aggregating with T-lymphocytes (*g*). All viable 7AAD^{-} cells are shown in these plots.

Immunohistochemistry was used to confirm that both CD1a^{+} dermal APCs (*h and j*) and CD1a^{+}CD207^{+} dermal Langerhans cells (*i-j*) formed close associations with dermal T-lymphocytes *in situ*. Epidermis is orientated upwards and blue represents DAPI staining of

cell nuclei. Scale bars represent 10μm. Data are representative of three independent experiments.

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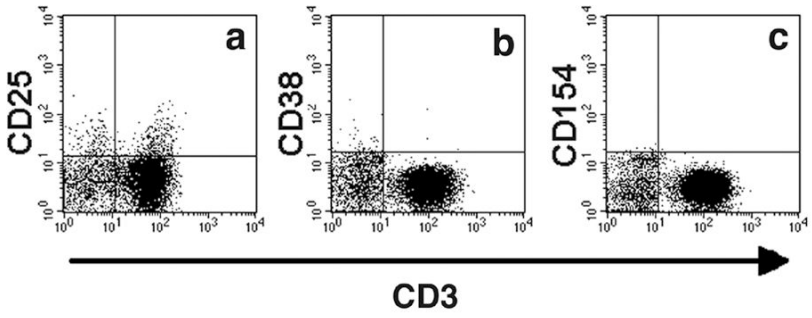

Figure 4. Assessing the activation status of T-lymphocytes in the human dermis

Single cell suspensions from human dermis were analyzed using flow cytometry. A small subset of dermal T-lymphocytes expressed low-level surface CD25 (*a*). The activation markers CD38 and CD154 were not detected on the surface of dermal T-lymphocytes (*b-c*). Data are representative of three independent experiments.

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