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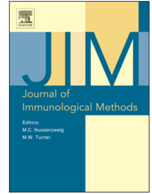

Research paper

Identification of skin immune cells in non-human primates

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ABSTRACT

The skin is a valuable target for vaccine delivery because it contains many immune cell populations, notably antigen presenting cells. Skin immune cells have been extensively described in mice and humans but not in non-human primates, which are pertinent models for immunological research in vaccination. The aim of this work was to describe immune cell populations in the epidermis, dermis and skin draining lymph nodes in cynomolgus macaques by a single 12-parameter flow cytometry protocol. Given that skin cells share several markers, we defined a gating strategy to identify accurately immune cells and to limit contamination of one immune cell population by another. The epidermis contained CD1a$^+$CD1c$^-$ Langerhans cells (LCs), CD3$^+$ T cells and putative NK cells. The dermis contained CD1a$^+$CD1c$^-$ cells, which were similar to LCs, CD1a$^+$CD1c$^+$ dermal dendritic cells (DDCs), CD163^{high}CD11b$^+$ resident macrophages, CD3$^+$ T cells and putative NK cells. The skin also contained CD66$^+$ polymorphonuclear cells in some animals. Thus, immune cell populations in the macaque are similar to those in humans despite some differences in phenotype. In skin draining lymph nodes, we identified migratory LCs, CD1a$^+$CD1c$^+$ DDCs and macrophages. The simultaneous identification of these different immune cells with one panel of markers avoids the use of large amounts of precious sample and may improve the understanding of immune mechanisms in the skin after treatment or vaccination.

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1. Introduction

The skin is made up of three layers and is an important part of organism defense because it acts as a physical barrier and has immunological properties (Kupper and Fuhlbrigge, 2004). The outer layer, the epidermis, is composed of 90% keratinocytes (Nestle et al., 2009) which are linked to corneocytes at the supra-layer by tight-junctions, which gives the skin its hermetic properties (Elias et al., 2001; Brandner et al., 2002; Morita et al., 2011). The underlying dermis provides the strength and elasticity of the skin through an abundant extracellular matrix rich in collagens, elastic fibers and reticulin (Shimizu, 2007). Finally, the innermost layer, the hypodermis, is mostly composed of adipocytes. The skin contains many immune cells including antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, as well as sentinel T cells that watch for potential injury or infection (Kupper and Fuhlbrigge, 2004; Nestle et al., 2009) and protect against pathogens (Jiang et al., 2012). DCs are an important heterogeneous population (Ju et al., 2010) that recognize various antigens (Ag) through the expression of pattern recognition receptors (Cerboni et al., 2013) and strongly activate and induce the differentiation of naïve T cells (Steinman and Witmer, 1978; Zaba et al., 2007; Iijima et al., 2011; van Spriel and de Jong, 2013). In response to Ag recognition, DCs activate, mature and migrate to draining lymph nodes (LNs), where they present Ag to T cells (Villablanca and Mora, 2008; Haniffa et al., 2012; Murakami et al., 2013). Several studies have demonstrated that the activation of DC subsets orients the immune system towards a Th1, Th2, Th17, Th22 or T regulator cell response (Jonuleit et al., 2000; Kapsenberg, 2003; Klechevsky et al., 2008; Jongbloed et al., 2010; Li et al., 2012; Seneschal et al., 2012). Consequently, the presence of DCs and the microenvironment generated at infected or vaccinated sites are two crucial variables involved in adaptive immune responses that are important to understand immunological mechanisms. For these reasons, the skin is an attractive organ to study immunity, auto-immune diseases or response to vaccination.

Several APCs, all of which express CD45 and HLA-DR, have been described in human skin (Valladeau and Saeland, 2005). Human Langerhans cells (LCs) are a population of DCs that express high levels of CD1a, CD207 and HLA-DR (Klechevsky et al., 2008; Fujita et al., 2009; Furio et al., 2010). These cells are located in the epidermis where they form a dense network with long dendrites between

Abbreviations: Ab, antibody; Ag, antigen; APC, antigen presenting cells; DC, dendritic cell; DDC, dermal DC; LC, Langerhans cell; LN, lymph node; Mac, macrophage; mDC, myeloid DC; mLC, migratory LC; Mono, monocytes; NHP, nonhuman primate; NK cells, Natural killer cells; pDC, plasmacytoïd DC; PMNs, polymorphonuclear cells; res, resident; vs, versus.

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Table 1
Flow cytometry panel to study immune cell populations in the skin and LNs of cynomolgus macaques.

Specificity	Fluorochrome	Clone	Supplier[a]	Quantity per sample
Live/Dead	UV450		Life Technologies	1 μl/ml
CD1a	AF-700[b]	O10	Dako	0.5 μg
CD11c	APC	S-HCL3	BD	2 μl
CD14	APC-H7	M5E2	BD	5 μl
CD66abce	FITC	TET2	Miltenyi	2 μl
CD1c	PE	AD5-8E7	Miltenyi	5 μl
CD11b	PE-Cy7	Bear 1	Beckman Coulter	2 μl
CD163	AF-594	GHI/61	BD	0.5 μg
CD123	AF-594	7G3	BD	1 μg
HLA-DR	PerCP	L243	BD	2 μl
CD45	V-450	DO58-1283	BD	2 μl
CD3[c]	V-500	SP34-2	BD	5 μl
CD8[c]	V-500	RPA-T8	BD	5 μl
CD20[c]	V-500	L27	BD	5 μl
CD207[d]	AF647	2G3	Baylor Institute	1 μg

[a] Life Technologies, Saint Aubin, France; Dako, Les Ulis, France; BD Biosciences, Le Pont de Claix, France; Miltenyi Biotec SAS, Paris, France; Beckman coulter, Villepinte, France; Baylor Institute for Immunology Research, Dallas, TX.
[b] Fluorochrome abbreviations: APC: allophycocyanin; FITC: Fluorescein isothiocyanate; PE: phycoerythrin; Cy7: Cyanine-7; PerCP: Peridin-chlorophyll; AF: Alexa-Fluor.
[c] LN samples were analyzed with three Ab (anti-CD3, −CD8, −CD20) coupled to the V500 fluorochrome whereas skin samples were analyzed with anti-CD3 V500 only.
[d] The CD207 Ab was used separately to verify the expression of langerin on DC subsets isolated by the 12-parameter panel.

keratinocytes (Pearton et al., 2010). The dermis contains CD1a$^+$CD14$^-$ dermal DC (DDCs) and CD1a$^-$CD14$^+$ DDCs, both of which express the myeloid DC markers CD11c and CD1c (Lenz et al., 1993; Nestle et al., 1993; Klechevsky et al., 2008; Furio et al., 2010). Several subsets of skin APCs express CD14, in particular monocytes and monocyte-derived APCs such as macrophages and some DC subsets. Two populations of CD141$^+$DDCs, with opposite functions depending on the expression of CD14, were described recently (Chu et al., 2012; Haniffa et al., 2012). CD141$^+$ CD14$^-$ DDCs strongly stimulate T cells (Haniffa et al., 2012), whereas CD141$^+$ CD14$^+$ DDCs are involved in skin tolerance and inhibit inflammation by inducing T regulatory cells (Chu et al., 2012). The dermis also contains a population of CD163$^+$ cells, corresponding to resident macrophages that also express CD14 (Zaba et al., 2007; Ochoa et al., 2008; Haniffa et al., 2009). In addition, normal skin contains CD3$^+$ T cells with homing specificities (Clark et al., 2006; Nestle et al., 2009). These populations have been largely described in humans (Clark et al., 2006; Zaba et al., 2007; Klechevsky et al., 2008; Ochoa et al., 2008; Chu et al., 2012; Haniffa et al., 2012) and in mice (Bursch et al., 2007; Henri et al., 2010; Chodaczek et al., 2012; Jiang et al., 2012; Gao et al., 2013; Kumamoto et al., 2013; Tamoutounour et al., 2013). Conversely, few studies have described these skin populations in non-human primates (NHP) (Romain et al., 2012; Epaulard et al., 2014), which are important models to study human infectious diseases (Gardner and Luciw, 2008) and responses to vaccine because of their close genetic relationship with humans (Herodin et al., 2005; Yan et al., 2011). Moreover, NHP models are essential to conduct invasive studies in the skin that would be impractical to perform in humans. Despite a few differences, leukocyte phenotypes are similar in humans and NHPs. Monoclonal antibodies directed against human markers often cross react with the simian counterpart of these molecules (Herodin et al., 2005).

The phenotypes of APCs isolated from the skin and the number of subpopulations identified is affected by the extraction protocol (Bond et al., 2009; Stoitzner et al., 2010; Chu et al., 2011). In some studies, immune cells are obtained by a migration process. In this procedure, the epidermis and dermis are enzymatically separated and are incubated in medium with or without cytokines (Klechevsky et al., 2008; Stoitzner et al., 2010). Immune cells are then retrieved in medium after several days of culture. This process is very efficient to obtain DCs, but cells with poor migratory properties such as tissue resident macrophages are not recovered (Haniffa et al., 2009). Alternatively, tissue digestion with enzymatic and mechanical disruption enables potentially all skin cell populations to be extracted in a short period of time (Zaba et al., 2007; Ochoa et al., 2008; Stoitzner et al., 2010). Consequently, this procedure avoids exposure to cytokines and in vitro migration, which activate recovered APCs (Zaba et al., 2007; Klechevsky et al., 2008).

In this study, we used a tissue digestion and mechanical disruption protocol to extract skin cell populations from 8-mm-diameter punch biopsies from the back of cynomolgus macaques. We used a 12-parameter flow cytometry panel of markers and a specific gating strategy to study skin immune cell populations present in healthy skin. The simultaneous analysis of 12 parameters compensated for the limited number of extracted cells and enabled skin-derived DCs in the dermis to be compared with those in the epidermis or skin draining LNs.

2. Material and methods

2.1. Animals

Twelve adult male cynomolgus macaques (*Macaca fascicularis*) imported from Mauritius and weighing 4–9 kg, were housed in CEA

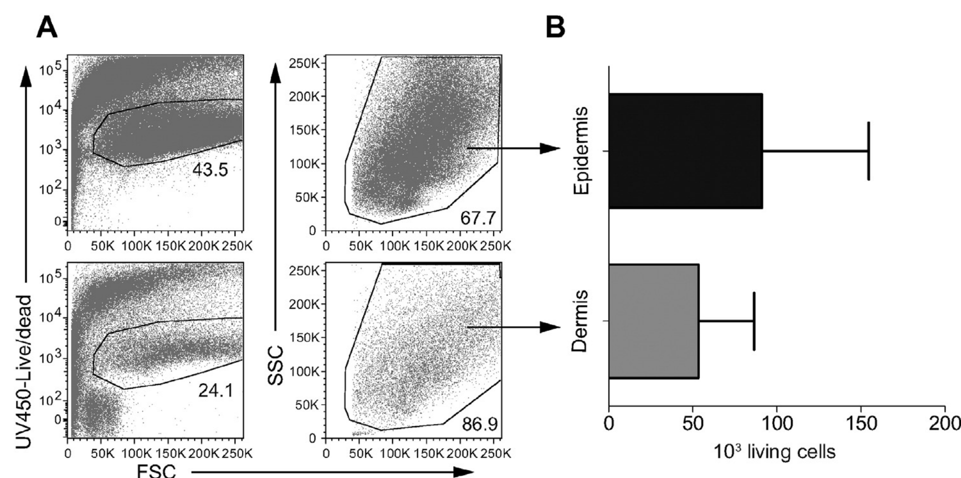

Fig. 1. Quantification of cells extracted from skin biopsies of 8 mm in diameter. The epidermal and dermal sheets were separated by dispase. The sheets were then incubated with collagenase (dermis) or collagenase + trypsin (epidermis) and were dissociated mechanically to obtain cell suspensions. (A) Cell suspensions were analyzed by flow cytometry to assess the number of living cells per sheet. (B) Living cells extracted from each sheet were quantified and plotted as the mean ± SD (n = 45).

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