



Research paper

A novel variation of the mixed lymphocyte reaction for measuring T cell responses to skin-specific antigens of pigs

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ABSTRACT

Skin and vascularized composite allografts (VCA) containing skin are transplanted to restore form and function of tissues after major injuries. Skin has long been recognized as being particularly immunogenic, causing high risk of rejection and immune sensitization. Due to skin-specific antigens, donor skin is often rejected even in animals that are tolerant of the remaining donor tissue. To study the reaction of lymphocyte subsets against these minor and/or tissue-specific skin antigens in swine made tolerant to allogeneic donors through hematopoietic stem cell transplants (HSCT), we developed a skin-adapted variation of the mixed lymphocyte reaction (MLR). We processed porcine skin into single cell suspensions to be used as stimulators. Peripheral blood mononuclear cells were used as responders. We first optimized the concentrations of skin stimulators to achieve T cell proliferation with minimal self-background reactivity. The assay was then tested in two pigs that had received combined VCA/HSCT. The first pig had not rejected any part of the VCA, and the second pig was actively rejecting the epidermis of the VCA at the time of the assay. Despite lack of anti-donor MLR responses against donor lymphocytes in the peripheral blood in either animal, the second pig demonstrated a specific response against donor skin cells. Our results suggest that the assay will be useful to study recipient sensitization against skin antigens, even in otherwise tolerant animals. This assay may have both diagnostic and therapeutic implications for immune responses specific to the skin.

1. Introduction

Vascularized composite allografts (VCA) containing skin are transplanted to restore form and function of tissues after major injuries. The number of VCA transplants performed clinically has gradually increased over the past few decades, since the first successful upper extremity transplantation was performed in 1998 (Kueckelhaus et al., 2016; Wells et al., 2022), and it is now considered standard of care in the United States (Department of Health and Human Services, 2011) and the European Union (Thuong et al., 2019). In allotransplantation, one must overcome the immune response from the recipient directed against the graft.

Skin has long been recognized to be particularly immunogenic (Murray, 1971; Steinmuller, 1998). Due to variations in skin-specific antigens, donor skin is often rejected even in animals that are otherwise tolerant of the remaining donor tissue (Fuchimoto et al., 2001;

Shanmugarajah et al., 2017; Weiner et al., 2012). Therefore, skin-containing grafts often require higher doses of immunosuppression than most solid organ transplants (Steinmuller, 1998). Immunosuppressive agents carry risk of morbidity (Landin et al., 2012; Wells et al., 2022) and, despite their use, both acute and chronic rejection of VCA transplants have been reported (Wells et al., 2022). In an attempt to alleviate this problem through induction of immunologic tolerance, we have developed a porcine model of combined hematopoietic stem cell transplant (HSCT) and VCA from the same donor. There are many similarities in the structure (Khiao In et al., 2019) and immunity (Summerfield et al., 2015) of pig and human skin, which makes the pig a good model for such experimental studies. To study the reaction of lymphocyte subsets against these tissue-specific skin antigens, we developed a skin-adapted variation of the mixed lymphocyte reaction (MLR). We report here the methodology and effectiveness of this *in vitro* assay and propose this assay as a means of assessing the recipient's

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response to donor skin-specific antigens within the graft, which may be used as a tool to study the mechanisms of tolerance and/or rejection of skin allografts.

2. Materials and methods

2.1. Animals and tissue collection

This experiment was performed as a part of separate IACUC protocols AC-AABI0555, AC-AABU2660, AC-AABN1550, and in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011). We used Sachs miniature swine, which have been inbred into strains bearing defined class I and class II swine leukocyte antigens (SLA, the pig equivalent of the major histocompatibility complex), such that transplants can be performed across defined genetic mismatches. The immunogenetic characteristics of Sachs miniature swine have been described previously (Sachs et al., 1976). A summary of the major histocompatibility complexes (MHC) haplotypes is presented in Appendix A.

For the initial development of the lymphocyte *versus* skin reaction (LSR) assay, we collected blood and skin from four pigs undergoing unrelated terminal procedures, and we collected blood from an additional two swine from our breeding herd located at Accuro Farm (Chazy, NY). The assay was first tested on non-experimental animals to establish the optimal ratio of cells and media using skin from swine SLA-matched and SLA-mismatched to the responder (pigs 1–6). We then attempted our methods on experimental animals who had previously received hematopoietic stem cell transplants (HSCT) and vascularized composite allografts (VCA) (pigs 7 and 10) from donor pigs (pigs 8 and 11, respectively). Pig 7 had not rejected any part of the VCA after transplantation, and pig 10 was actively rejecting the epidermis of the VCA at the time of the assay. Despite skin rejection in one of the experimental animals, both pigs had stable mixed hematopoietic chimerism and no donor-specific T cell responses in peripheral blood. Skin and peripheral blood mononuclear cells (PBMCs) from the HSCT/VCA donors (pigs 8 and 11) and third party animals (pigs 6 and 9) were used as stimulators. Additional stimulators included PBMCs from a pig with the identical SLA to that of the two donors (pig 12). In the present report we describe the *in vitro* results obtained using this assay, while the *in vivo* results of these VCA transplants will be published elsewhere. Animal characteristics are presented in Table 1. For reference, historical data with mixed lymphocyte reaction (MLR) assay responses for the relevant SLA haplotypes of strains of Sachs miniature swine SLA haplotypes (Sachs et al., 1976) used in this publication are presented in Appendix B.

2.2. Assay media and wash buffer

Assay medium was prepared by combining 33 mL Gibco™ Porcine Serum (Fisher Scientific, Waltham, MA), heat-inactivated in a 56 °C water bath for 30 min, with 500 mL Gibco™ AIM-V Medium (Fisher Scientific, Waltham, MA), and sterile filtering through Nalgene™ Rapid-Flow™ 0.2 µm Filter Units (Fisher Scientific, Waltham, MA). The media was kept at 4 °C.

Wash buffer was prepared by combining 890 mL deionized water, 100 mL Hanks' Balanced Salt Solution (HBSS) (10×) no phenol red and 10 mL Metal mix (Thermo Scientific, Waltham, MA), 1 g Sodium Azide (Acros Organics, Antwerp, Belgium), 1 g Bovine Serum Albumin Fraction V (Roche, Basel, Switzerland) and 210uL Sodium Hydroxide Solution 1 N (Fisher Scientific, Waltham, MA).

2.3. PBMC isolation

PBMCs were isolated from freshly collected blood. The collected blood was heparinized, and mononuclear cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (TONBO Biosciences, San Diego, CA). The cells were washed with Hanks'

Table 1

Breed and swine leukocyte antigen (SLA) haplotypes for the pigs used to develop the Lymphocyte vs Skin Reaction assay. Note: Both experimental VCA transplants were across a single Class I haplotype difference.

Pig	Breed	SLA haplotype*	Use in MLR and LSR assays
1	Duroc, landrace, Yorkshire cross	Unknown	Responder in experiment 1
2	Sachs miniature swine	HH	Stimulator in experiment 1
3	Sachs miniature swine	GD	Responder and stimulator in experiment 1
4	Sachs miniature swine	CC	Responder and stimulator in experiment 2
5	Sachs miniature swine	DD	Responder in experiment 2
6	Yucatan	Unknown	Responder in experiment 2 and stimulator in experiment 3
7	Sachs miniature swine	DD	Responder and stimulator in experiment 3
8	Sachs miniature swine	GD	Stimulator in experiment 3
9	Sachs miniature swine	AA	Stimulator in experiment 3
10	Sachs miniature swine	GG	Responder and stimulator in experiment 3
11	Sachs miniature swine	GD	Stimulator in experiment 3
12	Sachs miniature swine	GD	Stimulator in experiment 3

* For details of SLA Haplotypes and genetic differences of the strains, see Appendix A.

Balanced Salt Solution (HBSS) (Corning, Corning, NY). Thereafter, the remaining red blood cells were lysed by incubating with ACK Lysing Buffer (Fisher Scientific, Waltham, MA) for 5 min, and the cell suspension was washed again with HBSS.

2.4. Mixed lymphocyte reaction

For MLR assays, responder PBMCs were resuspended in HBSS at a concentration of 1×10^6 cells /mL in 50 mL conicals. The cells were stained with 10 µL CellTrace™ CFSE (Invitrogen, Carlsbad, CA), at a concentration of 28 µg/mL, per 1 million cells. The cells were incubated for 7 min at room temperature in the dark. After incubation, the tubes were quenched by filling to 50 mL with media and placed on ice for 5 min and then centrifuged at 1650 rpm for 5 min at 4 °C. The tubes were washed one more time with media and centrifuged at 1650 rpm for 5 min at 4 °C. The cells were counted and resuspended in assay media at 1.5×10^6 cells per mL. Stimulator cells were resuspended in 1 mL HBSS per 30 million cells in 50 mL conicals, and 1 µL of Violet Proliferation Dye 450 (BD Biosciences, Franklin Lakes, NJ), at a concentration of 0.38 mg/mL, per mL was added. The cells were incubated in 37 °C water bath for 12 min and thereafter washed twice by quenching the tubes with media and centrifuged at 1650 rpm for 5 min at 4 °C. The cells were counted and resuspended in media at 2×10^6 cells per mL and irradiated with 30 Gy X-ray using an X-Rad320 (Precision X-ray, Madison, CT) Responders and stimulators were plated in triplicates in 96-well flat bottom plates (CELLTREAT, Pepperell, MA), assay media was used as negative control, and 1 µL of phytohemagglutinin-P (PHA) (InvivoGen, San Diego, CA), at a concentration of 5 mg/mL, was added into positive control wells. Each well had a total volume of 250 µL and the plates were incubated at 37 °C in 5 %CO₂ for six days.

2.5. Skin cell isolation

Hair was trimmed off using a clipper and the skin thereafter sprayed with 1 % iodine. 6 mm punch biopsies were collected with sterile instruments into MACS® Tissue Storage Solution (Miltenyi Biotec,

Bergisch Gladbach, Germany). Biopsies were put inside a petri dish and subcutaneous tissue trimmed off. GentleMACS™ C Tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) were prepared according to the manufacturer's instruction for the Whole Skin Dissociation Kit, Human (Miltenyi Biotec, 130–101-540, Bergisch Gladbach, Germany). This kit has previously been used for isolation of pig skin cells for single-cell RNA sequencing (Han et al., 2022). The skin from two 6 mm punch biopsies were placed in one C Tube and incubated in a 37 °C water bath for 3 h. After the incubation, samples were diluted with 0.5 mL of cold media. The C Tubes were run on a GentleMACS™ Tissue Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) using the *h_skin_01* program. The tubes were thereafter detached, and after a short centrifugation step the cell suspension was applied on a 70 µm Advanced Cell Strainer (Genesee Scientific, El Cajon, CA), placed on a 50 mL conical. The filter was washed with 4 mL media and the tubes were quenched to 50 mL with media. The cell suspension was centrifuged at 1650 rpm for 5 min at 4 °C, the supernatant was poured off and the pellet ratcheted. The tubes were again quenched to 50 mL with media and incubated with 200 µL of Amphotericin B (Fisher Scientific, Waltham, MA) and 500 µL of Penicillin-Streptomycin (Sigma-Aldrich, Saint Louis, MO) for 30 min in 4 °C. After incubation, the cell suspension was centrifuged at 1650 rpm for 5 min at 4 °C, supernatant was poured off and the pellet ratcheted. The cells were counted with an automated cell counter (Countess 3FL, Invitrogen, Carlsbad, CA) and resuspended in media at concentrations ranging from 6.3×10^4 /mL to 2×10^6 /mL.

To be able to assess reactivity against donor skin for the experimental animals at a later time point, split thickness skin grafts (STSGs) were harvested from donor animals immediately after euthanasia and frozen. Hair was trimmed off using a clipper and the skin thereafter sprayed with 1 % iodine. STSGs (0.6 mm) were harvested with a Zimmer dermatome and briefly kept in cold saline before freezing. STSGs were frozen in pieces up to 4"x2". Each skin piece was placed in between two pieces of N-TERFACE® Interpositional Surfacing Material (Winfiled Laboratories, Richardson, TX), rolled up and placed in a 8 mL tube. The tubes were filled with medium containing 50 % RPMI-1640 (Cytvia, Marlborough, MA) and 50 % Cryoprotective Medium (Lonza, Basel, Switzerland). The vials were then frozen to –80 °C using a CryoMed Controlled Rate Freezer (Thermo Fisher Scientific, Waltham, MA) and thereafter transferred to a –80 °C freezer. For STSG thawing, medium was prepared by combining 33 mL Gibco™ Porcine Serum (Fisher Scientific, Waltham, MA), heat-inactivated in a 56 °C water bath for 30 min, with 500 mL Gibco™ AIM-V Medium (Fisher Scientific, Waltham, MA), and sterile filtering through Nalgene™ Rapid-Flow™ 0.2 µm Filter Units (Fisher Scientific, Waltham, MA). 1 mL of Amphotericin B (Fisher Scientific, Waltham, MA) and 2.5 mL of Penicillin-Streptomycin (Sigma-Aldrich, Saint Louis, MO) was added into 250 mL of the medium. STSGs were briefly thawed until the content was detached from the wall of the tube and then transferred to the antimicrobial agent containing medium. The bottle was briefly swirled and the skin thereafter transferred to medium without antimicrobial agents. 6 mm punch biopsies were collected from thawed skin and skin cells thereafter isolated as described above.

2.6. Lymphocyte vs skin reaction assay

Skin cells were isolated as described above, and the cell suspensions were irradiated with 30 Gy X-ray using an X-Rad320 (Precision X-ray, Madison, CT) to be used as stimulators in the LSR assay.

PBMCs were used as responders and first resuspended at 1×10^6 cells per mL in 50 mL conicals. The cells were stained with 10 µL CellTrace™ CFSE (Invitrogen, Carlsbad, CA), at a concentration of 28 µg/mL, per 1 million cells. The cells were incubated for 7 min in room temp in the dark. After incubation, the tubes were quenched to 50 mL with media and placed on ice for 5 min and then centrifuged at 1650 rpm for 5 min at 4 °C two times. The cells were counted and resuspended in media at 1.5 million cells per mL.

Responders and stimulators were plated in triplicate in 96-well flat bottom plates (CELLTREAT, Pepperell, MA), assay media was used as negative control and 1 µL of PHA (InvivoGen, San Diego, CA), at a concentration of 5 mg/mL, was added into positive control wells. Three wells were used to get a sufficient number of cells for FACS analysis, and the cells were pooled at takedown. Each well had a total volume of 250 µL and the plates were incubated at 37 °C in 5 %CO₂ for six days.

2.7. Staining of cells from MLR and LSR assays

After six days, the cells from MLR and LSR assays were harvested into FlowTubes™ (MTC Bio, Metuchen, NJ) and quenched with HBSS. The tubes were centrifuged twice at 1650 rpm for 5 min at 4 °C. The tubes were stained with PE-Cy™7 Mouse Anti-Pig CD3e (Clone BB23-8E6-8C8, BD Pharmingen, Franklin Lakes, NJ), PerCP-Cy™5.5 Mouse Anti-Pig CD4a (Clone 74-12-4, BD Pharmingen, Franklin Lakes, NJ) and Alexa Flour® 647 Mouse Anti-Pig CD8a (Clone 76-2-11, BD Pharmingen, Franklin Lakes, NJ) and incubated for 30 min at 4 °C. After incubation, the tubes were quenched with FACS buffer and centrifuged at 1650 rpm for 5 min at 4 °C. Just before the samples were run on a 5Laser Cytex® Aurora (Cytex Biosciences, Fremont, CA), 10 µL of DAPI Staining Solution (Abcam, Cambridge, UK), diluted 1:1000 in wash buffer, was added into each tube.

2.8. Data analysis

Flow cytometry data was analyzed using FCS Express (De Novo Software, Pasadena, CA). Percentage proliferation was defined as percentage of CFSE low cells for live CD3+, CD3 + CD4 + CD8-, CD3 + CD4-CD8+ and CD3 + CD4 + CD8+ cells respectively. Gating strategy is presented in Appendix C.

2.9. Statistical analysis

Normal distribution was tested by Shapiro-Wilk test and the data was normally distributed. Differences between groups were tested with paired *t*-test. *P*-values <0.05 were considered significant. All statistical analyses were performed in GraphPad Prism 10.4.1.

3. Results

3.1. Skin cell isolation

From biopsies collected from normal skin, either under anesthesia or just after euthanasia, the cell yields averaged 6.2×10^5 cells per 6 mm biopsy (range 1.7×10^5 – 1.4×10^6). For biopsies collected from skin with active inflammation (from pig 10), the cell yield was 5.5×10^6 cells per 6 mm biopsy. For biopsies collected from thawed skin (from pigs no 8 and 9), the cell yield was 1.7×10^5 and 1.6×10^5 cells per 6 mm biopsy respectively.

3.2. Experiment 1 – stimulator titration

Experiment 1 was carried out to titrate the concentration of stimulators that would provide adequate stimulation, with minimal (<5 % CD3 proliferation) self-background reactivity. This experiment utilized normal animals with SLA disparate haplotypes. The first LSR assay was done with PBMCs from pig 1 (outbred) as responder and pig 2 (HH) skin cells as stimulators (see Table 1 and Appendix A for haplotype combinations). For this initial exploratory experiment, only one technical replicate was performed. Stimulators were plated at concentrations of 2×10^6 /mL, 1×10^6 /mL, 5×10^5 /mL and 2.5×10^5 /mL (Fig. 1A). These concentrations were selected based on our experience of lymphoid stimulator concentrations used in other MLR assays. In this initial assay, no proliferation was observed in wells containing the highest concentration of stimulators (5×10^5 – 2×10^6 cells/mL). In these wells, most

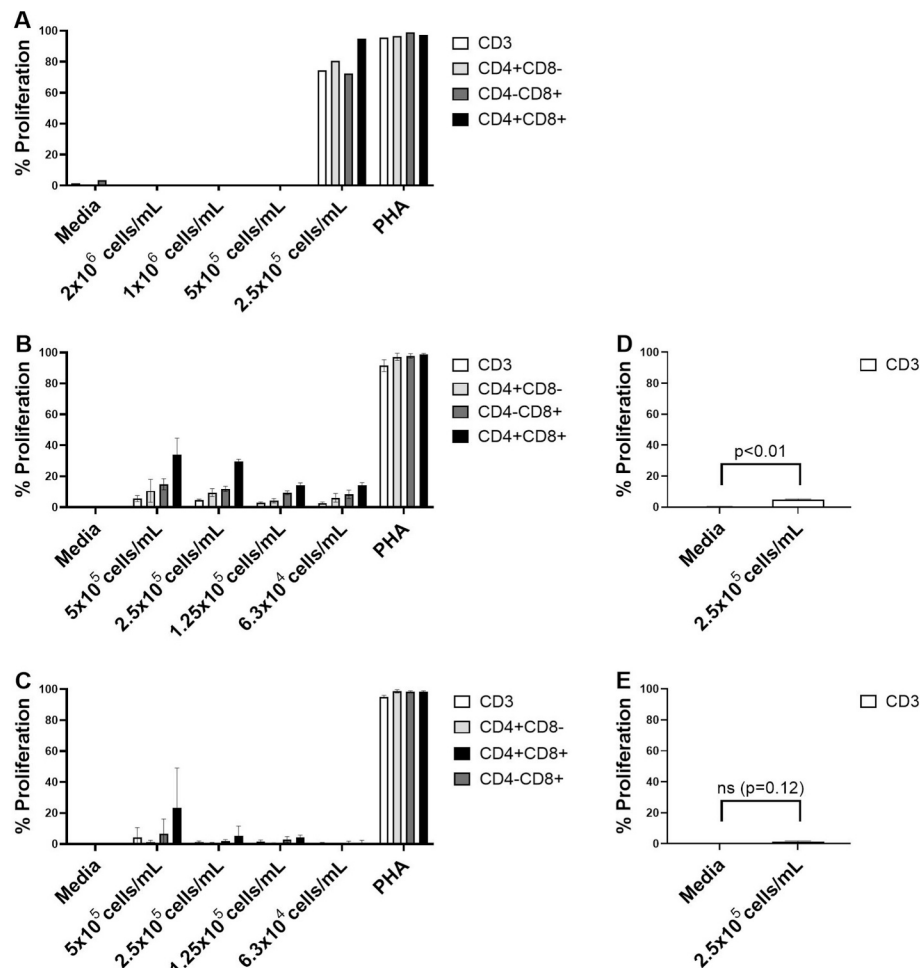


Fig. 1. Percent proliferation for PBMC responders to skin cell stimulators in a Leukocyte vs Skin Reaction Assays. Skin cell stimulator concentrations are indicated on x-axis. All assays included negative control media and positive control PHA. LSRs shown in B and C were plated with three technical replicates, bars represent mean and error bars represent standard deviation. A. First stimulator titration experiment with SLA mismatched pigs, pig 1 (outbred) as responder and pig 2 (HH) as stimulator. B. Second titration experiment with SLA mismatched pigs and decreased stimulator concentrations compared to A. Pig 1 (outbred) as responder and pig 3 (GD) as stimulator. C. LSR assay carried out with same pig (pig 3, GD) as responder and stimulator to assess self reactivity (self background) for the same concentrations as used in B. D. CD3 proliferation for 2.5×10^5 stimulator cells was significantly increased compared to media (paired t-test, $p < 0.05$) in the assay against the SLA mismatched pig. E. CD3 proliferation for 2.5×10^5 stimulator cells was not significantly different from media (paired t-test, $p = 0.12$) when the responder pig was plated against self.

cells appeared dead when run on the flow cytometer. Wells with 2.5×10^5 cells/mL, on the other hand, showed a good proliferation.

The second LSR assay was performed with PBMCs from pig 1 (outbred) and pig 3 (GD) as responders and skin cells from pig 3 (GD) as stimulator. This assay was performed to see at which concentrations there would be a response when SLA was mismatched, but with minimal response (self background) when pig 3 (GD) was used as both responder and stimulator. For this assay, three technical replicates were plated. Results are presented in Fig. 1B-C. The range of concentrations of stimulators were lowered to 5×10^5 /mL, 2.5×10^5 /mL, 1.25×10^5 /mL and 6.3×10^4 /mL. When a stimulator concentration of 5×10^5 /mL was used, variations in the results were observed with substantial self-skin reactivity and we decided not to continue with this concentration. For the other concentrations of stimulators (2.5×10^5 /mL, 1.25×10^5 /mL and 6.3×10^4 /mL) the results were consistent across assays. When using a stimulator concentration of 2.5×10^5 cells/mL there was proliferation for pig 1 (outbred) against pig 3 (GD), and minimal self-reactivity was observed when pig 3 (GD) was plated against self skin. Statistical analysis confirmed that the method was stable, see Fig. 1D-E. 2.5×10^5 cells/mL was therefore the concentration selected for further experiments. When stimulators were plated at 1.25×10^5 cells/mL and 6.3×10^4 cells/mL,

only a slight proliferation was seen, and these two lowest concentrations were considered to be too low for further study.

3.3. Experiment 2 – reproducibility of results

To confirm that plating 2.5×10^5 stimulator cells/mL, as indicated in experiment 1, gave consistent stimulation additional LSR assays were plated with SLA mismatched pigs, see Fig. 2. Pig 5 (DD) and pig 6 (outbred) were used as responders and pig 4 (CC) was used as stimulator. To ensure also that self reactivity was minimal ($< 5\%$ CD3 proliferation), one LSR was plated with the same pig (pig 4, CC) as responder and stimulator. The control assay with PBMCs and skin from the same pig showed minimal self background reactivity, while assays using PBMCs from the SLA disparate pigs (DD against CC and outbred against CC) showed good stimulation, which was consistent with the findings in experiment 1.

3.4. Experiment 3 – experimental application

After establishing the concentration of stimulators that gave a consistent proliferation with minimal self background, the assay was

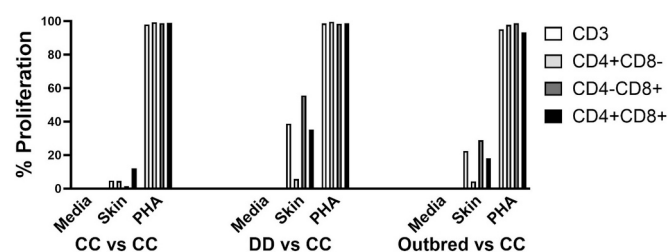


Fig. 2. Percent proliferation for PBMC responders from pig 4 (CC), pig 5 (DD) and pig 6 (outbred) plated against pig 4 (CC) skin cells stimulators in Leukocyte vs Skin Reaction Assays. Stimulator concentration of 2.5×10^5 was used to confirm good proliferation when DD and Outbred were plated against CC, but showed minimal self background when CC was plated against CC (self). Negative control = media and positive control = PHA.

tested with two experimental pigs that had received combined HSCT and VCA transplantation. The HSCT/VCA recipients, pig 7 (DD) and pig 10 (GG) had a single SLA Class I mismatch from their GD donors (pigs 8 and 11), see Table 1 and Appendix A. Both recipient pigs were tolerant to donor MHC as demonstrated by no reaction in MLR assays but differed in their clinical response to the VCA skin. Pig 7 (DD) had not rejected any part of the VCA including the skin portion, while pig 10 (GD) was actively rejecting the skin portion of the VCA at the time of the LSR assay. In the first assay, PBMCs from the recipient pig 7 (DD) were used as responders. This pig had received an HSCT with concurrent VCA transplant 175 days prior to the assay and had not shown any signs of rejection. For stimulators, self (DD) skin cells, VCA skin cells, thawed donor skin cells, and third-party AA (pig 9) skin cells were used (Fig. 3). In this assay PBMCs from pig 7 (DD) showed no reactivity against self, VCA or donor skin, while there was a substantial response against third-party skin, confirming *in vitro* tolerance to donor skin specific antigens. In the second assay, PBMCs from pig 10 (GG), also differing from its donor by a single Class I haplotype, were used as responders (Fig. 4A). This pig had received HSCT with concurrent VCA transplant 100 days prior to the assay and was actively rejecting the skin portion of the VCA. For stimulators, self skin cells and VCA skin cells were used. Additionally, an MLR with PBMCs from the recipient pig 10 (GG) as responders was plated, using self PBMCs, GD donor (pig 11) PBMCs, donor SLA-matched GD (pig 12) PBMCs, and third party PBMCs from an outbred pig (pig 6) as stimulators (Fig. 4B). PBMCs from pig 10 (GG) showed no response against self skin in the LSR assay, while there was a substantial response against skin cells isolated from the VCA (Fig. 4A) while simultaneously demonstrating hyporesponsiveness against PBMCs from the VCA donor and donor SLA-matched pig (Fig. 4B). We concluded from these data that the LSR assay was detecting sensitization against skin specific antigens in this model.

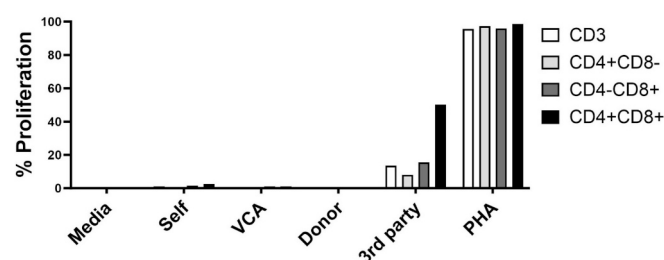


Fig. 3. Percent proliferation for pig 7 (DD) PBMC responders to skin cells stimulators from self skin (DD), GD VCA skin (pig 8), thawed GD donor skin (pig 8) and third party AA (pig 9) skin in a Leukocyte vs Skin Reaction Assay. Negative control media and positive control PHA. Pig 7 (DD) was not rejecting any part of the VCA, including skin, at the time of the assay.

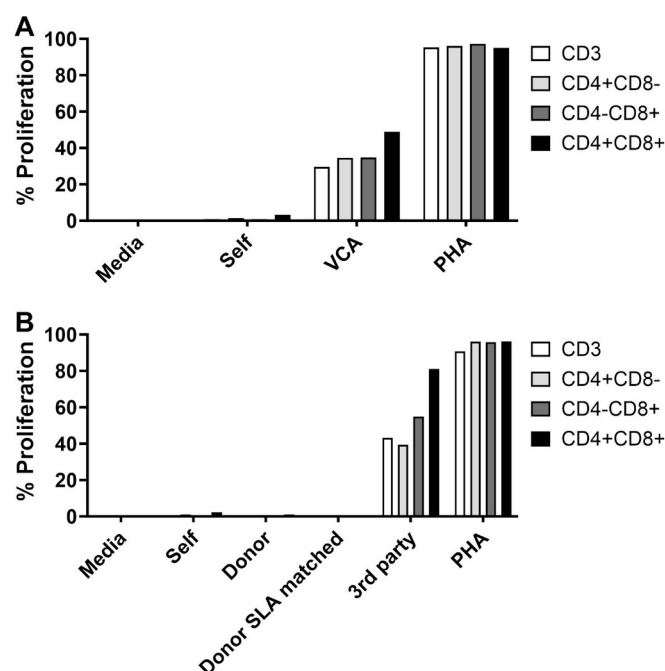


Fig. 4. LSR (A) and peripheral blood MLR (B) assays with PBMCs from pig 10 (GG) as responder. This GG pig was actively rejecting the skin portion of the VCA at the time of the assay. As stimulators in the LSR (A), self skin (GG) and VCA skin (GD). As stimulators in the MLR (B) self PBMCs (GG), donor GD (pig 11) PBMCs, donor SLA matched GD (pig 12) PBMCs and third party PBMCs from an outbred pig (pig 6). Negative control media and positive control PHA.

4. Discussion

We present a novel *in vitro* method for studying porcine lymphocyte reactions against tissue-specific skin antigens. Skin is recognized as being particularly immunogenic (Murray, 1971; Steinmuller, 1998) and the variations in skin-specific antigens usually causes donor skin to be rejected even in animals that are otherwise tolerant of the remaining donor tissue (Fuchimoto et al., 2001; Shanmugarajah et al., 2017; Weiner et al., 2012). This poses a particular problem for skin-containing VCA. Studying the reactions of lymphocytes against skin antigen by *in vitro* assays could contribute to the research field aiming to improve treatment strategies for this patient group.

We describe here the development of an *in vitro* assay in which responses of recipient lymphocytes to skin antigens can be assessed for this purpose. We demonstrate that this assay can be performed successfully and that it can provide insight into recipient responses to tissue-specific donor antigens that would not be detectable by standard MLR assays. When we titrated the concentrations of stimulators for the assay, we set the highest concentration to 2×10^6 cells per mL based on our experience with MLRs using PBMCs. However, at this concentration, and at 1×10^6 and 5×10^5 cells per mL, we did not see any proliferation and the cells appeared dead when run on the flow cytometer. Using stimulators at 2.5×10^5 cells per mL worked well and gave consistent stimulation. A likely explanation for the need for lower concentrations of stimulators when using skin cells is that the skin cells provide stronger stimulation than PBMCs due to dendritic cells in the skin cell suspension expressing MHC class II. The responders may therefore have been overstimulated at the higher concentrations of stimulators tested, causing nutrients in the media to be depleted more quickly. Finally, epidermis and dermis primarily consist of keratinocytes, dendritic cells and fibroblasts, all of which are larger cells than lymphocytes. The larger volume of cells in the wells compared to MLR assays may also have contributed to decreased survival of responders at the higher concentrations of stimulators tested.

Overall, our new assay evaluates recipient responses to donor skin-specific minor antigens that are not expressed on hematopoietic cells. Minor histocompatibility antigens have been shown to consist of peptides of allelic proteins within a species that are presented to T cells by MHC and can cause rejection even between MHC-identical donor-recipient pairs (Wallny and Rammensee, 1990). Although the different lines of Sachs miniature swine have been bred for homozygosity of MHCs, minor antigens vary between individuals in the herd. In the present experiment, we showed that even pigs that were tolerized to donor MHC by bone marrow transplantation may still have antigenic responses to donor skin cells bearing skin-specific antigens and reject the skin part of a VCA graft, as previously hypothesized in studies from our laboratory (Fuchimoto et al., 2001). This does not abrogate tolerance; the recipient who was tolerant of bone marrow and dermis but rejected VCA skin remained tolerant of donor bone marrow and dermis *in vivo* and did not respond to donor PBMC *in vitro* in a conventional MLR. However, the animal responded specifically to donor skin cells in the LSR assay. Thus, the importance of the LSR assay is that, unlike our conventional MLR using PBMCs, it is the only modality that shows the immune response to skin-specific antigens *in vitro*. Our data therefore provide a mechanistic confirmation of the hypothesized explanation for why HSCT did not induce tolerance to skin from the same donor in previous studies (Fuchimoto et al., 2001; Ildstad et al., 1985). We hope that this new assay will be useful for other experiments requiring analysis of recipient immune responses to skin-specific antigens.

5. Conclusion

In summary, we present a method to isolate porcine skin cells to be used as stimulators in a novel assay that can be used to study peripheral blood lymphocyte reactions against skin antigens. We have demonstrated that the LSR assay specifically recognized lymphocyte reactions against skin antigens when used to study a recipient that was sensitized against skin antigens by rejecting the skin component if a VCA *versus* a similarly prepared animal that had not rejected its skin graft. In both animals standard MLR assays, using only donor PBMCs but not skin stimulators, did not show any reactivity. The assay should be able to be combined with other methods, such as cell sorting, for mechanistic

studies. We leave open the possibility that this assay may even be used as a diagnostic tool for clinical VCA grafts containing skin.

CRediT authorship contribution statement

Elin Manell: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M. Esad Gunes:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Philip Jordache:** Writing – review & editing, Investigation. **Satyajit Patwardhan:** Writing – review & editing, Investigation. **Julie Hong:** Writing – review & editing, Investigation. **David Sachs:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Joshua Weiner:** Writing – review & editing, Supervision, Conceptualization.

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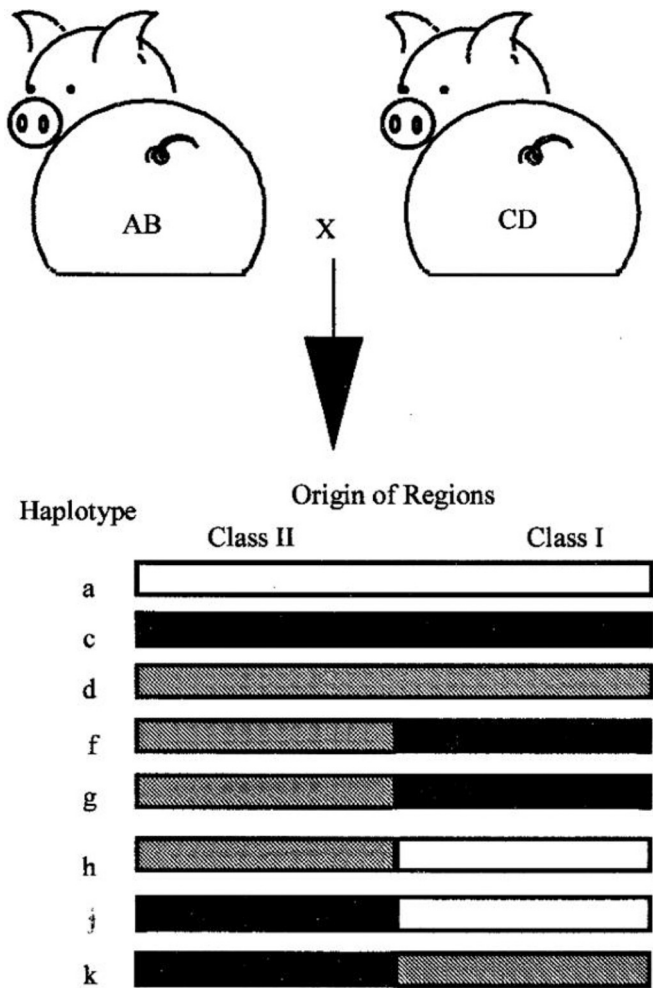
Declaration of competing interest

None.

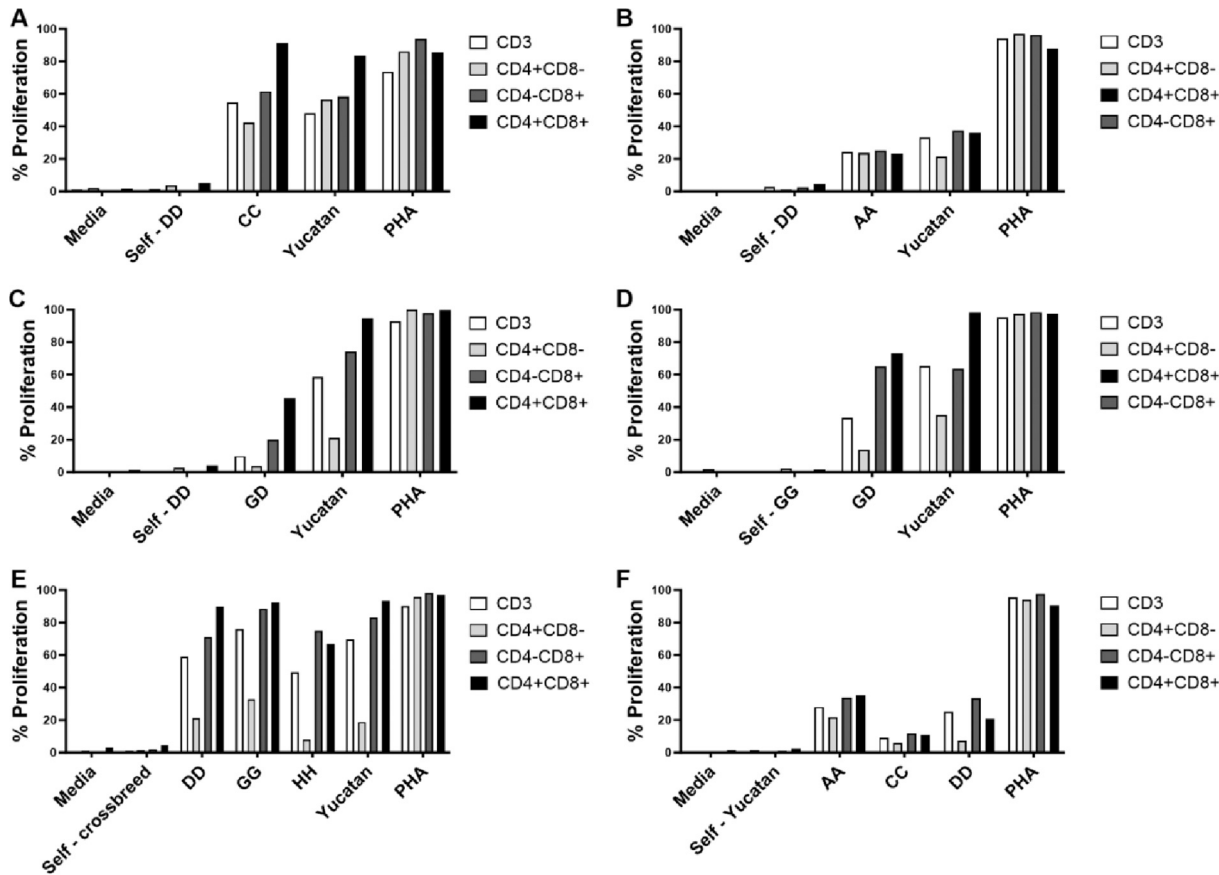
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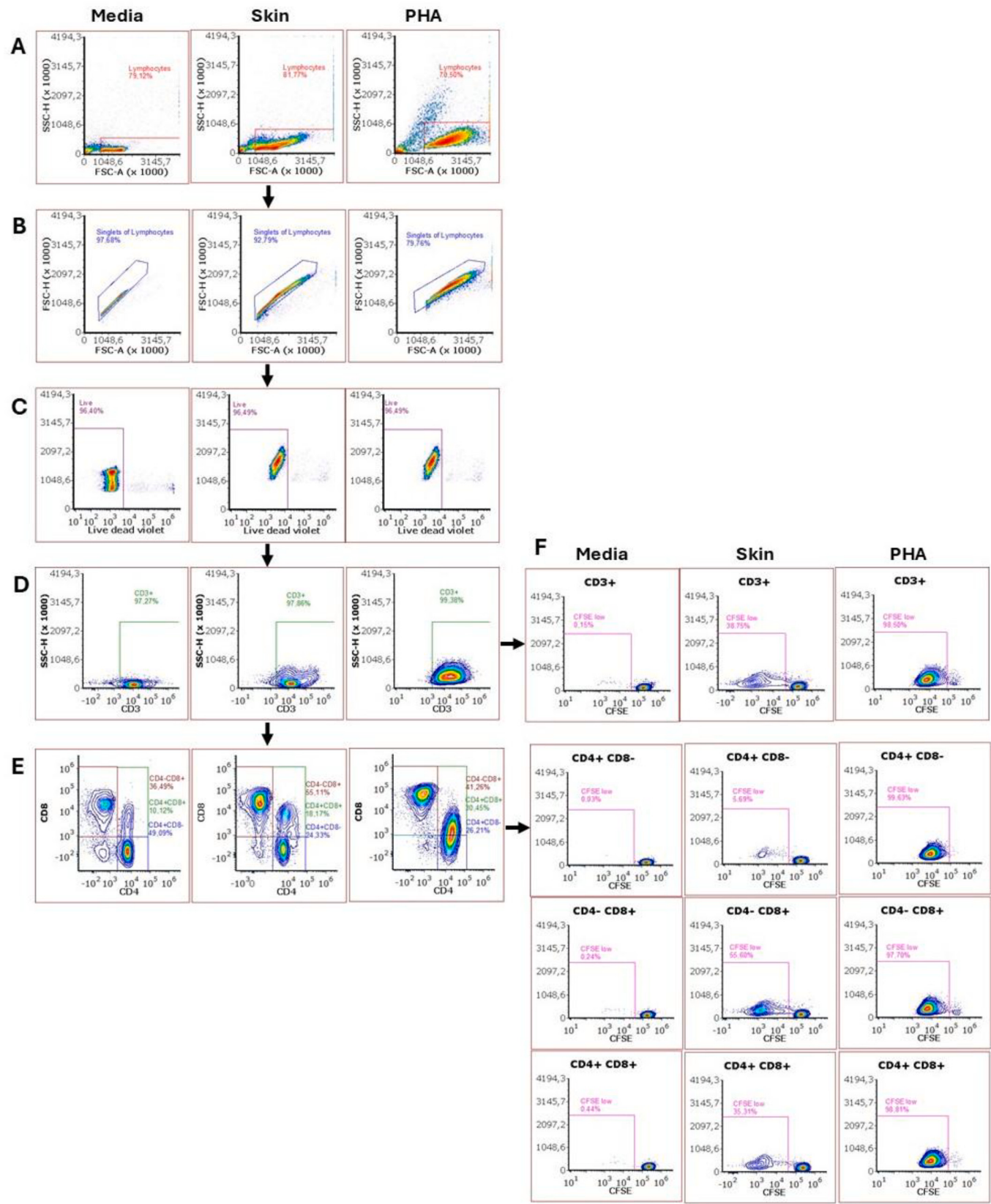
Appendix A. Haplotypes (a-k) of MHC Class I (a,c,d) and Class II (a,c,d) alleles of Sachs Miniature Swine. For example, a pig with SLA haplotype GD will have MHC Class I CD alleles and MHC Class II DD alleles



Appendix B. Historical data showing mixed lymphocyte reaction assay responses for breeds and Sach's miniature swine, swine leukocyte antigen haplotypes used in the development of the LSR assay. A. Sach's miniature swine responder DD plated against CC. B. Sach's miniature swine responder DD plated against AA. C. Sach's miniature swine responder DD plated against GD. D. Sach's miniature swine responder GG plated against GD. E. Responder crossbred pig (Duroc, Landrace, Yorkshire) plated against Sachs miniature swine DD, GG and HH. F. Responder Yucatan pig plated against Sachs miniature swine AA, CC and DD. In all assays, media was used as negative control, and positive controls were 3rd party pig of Yucatan breed and/or phytohemagglutinin-P (PHA)



Appendix C. Gating strategy for LSR assay with media, skin and phytohemagglutinin-P (PHA). First lymphocytes are selected (A), thereafter singlets (B), live cells (C), and CD3+ cells (D). The CD3+ cells are subdivided (E) into CD4 + CD8-, CD4-CD8+ and CD4 + CD8+ cells. Proliferation is assessed by percentage of CFSE low cells (F) for CD3+, CD4 + CD8-, CD4-CD8+ and CD4 + CD8+ cells



Data availability

Data will be made available on request.

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