Local immune response against larvae of Rhipicephalus microplus

Journal:	*Parasite Immunology*
Manuscript ID	PIM-2017-0109.R1
Manuscript Type:	Original Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Constantinoiu, Constantin; James Cook University, School of Veterinary and Biomedical Sciences Lew-Tabor, Alicja; The University of Queensland, QAAFI Jackson, Louise; Queensland Department of Agriculture and Fisheries, Biosecurity Science Laboratories Jorgensen, Wayne; Queensland Department of Agriculture and Fisheries, Agriculture and Fisheries Piper, Emily; Zoetis Genetics, Genetics Mayer, David; Queensland Department of Agriculture and Fisheries, Agriculture and Fisheries Johnson, Linda; University of Colorado Hospital, Cancer center Venus, Bronwyn; University of Queensland, Queensland Alliance for Agriculture & Food Innovation Jonsson, Nicholas; University of Glasgow, College of Medical, Veterinary and Life Sciences
Key Words:	Cell mediated immunity < Immunological terms, IFAT < Tools and techniques

Local immune response against larvae of the cattle tick (*Rhipicephalus (Boophilus) microplus)* in Santa Gertrudis cattle with low and high levels of tick resistance

Immune response against *Rhipicephalus microplus*

Constantinoiu, Constantin[1,2,3 *], Lew-Tabor, Ala[1,2,4], Jackson, Louise[1,2], Jorgensen, Wayne[1,2,3], Piper, Emily[1,3], Mayer, David[2], Johnson, Linda[5], Venus, Bronwyn.[2,3], Jonsson, Nicholas[1,3]

[1] Cooperative Research Centre for Beef Genetic Technologies, Armidale, Australia, 2351

[2] Department of Primary Industries and Fisheries, GPO Box 46, Brisbane, Queensland, Australia, 4001

[3]The University of Queensland, School of Veterinary Science, Gatton, Queensland, Australia, 4343

[4]Centre for Comparative Genomics, Murdoch University, Perth, Western Australia, Australia, 6150

[5]James Cook University, College of Public Health, Biomedical and Veterinary Sciences, 1 Solander Drive, Townsville, Queensland, Australia, 4811

* Corresponding author.

Present address

Constantinoiu, Constantin: College of Public Health, Biomedical and Veterinary Sciences, James Cook University, 1 Solander Drive, Townsville, Queensland, Australia, 4811

Lew-Tabor, Ala: Queensland Alliance for Agriculture & Food Innovation, The University of Queensland, 306 Carmody Rd., Blg 80, St. Lucia, Queensland, Australia 4067

Jackson, Louise: Queensland Department of Agriculture and Fisheries, Biosecurity Sciences Laboratory, Coopers Plains, Queensland, Australia 4108

Jorgensen, Wayne: Queensland Department of Agriculture and Fisheries, Ecosciences Precinct 2.A.West, 41 Boggo Road, Dutton Park, Queensland, Australia 4102

Piper, Emily: Zoetis Inc., 333 Portage St, Kalamazoo MI 49007, USA

Mayer, David: Queensland Department of Agriculture and Fisheries, Ecosciences Precinct 2.A.West, 41 Boggo Road, Dutton Park, Queensland, Australia 4102

Johnson, Linda: University of Colorado, Anschutz Medical Campus, Mail Stop 8104; 12800 E. 19th Ave, Room 5114, Aurora, CO 80045 USA

Venus, Bronwyn: Queensland Alliance for Agriculture & Food Innovation, The University of Queensland, 306 Carmody Rd., Blg 80, St. Lucia, Queensland, Australia 4067

Jonsson, Nicholas: The University of Glasgow, Institute of Biodiversity, Animal Health and Comparative Medicine, Glasgow, Scotland, UK.

Disclosures: none

Acknowledgments

Thanks to Tom Connolly and Matt Verri for assistance with animal trials, to Ralph Stutchbury for the provision of ticks, to Laercio Porto for carrying out the biopsies, special thanks to John Molloy for review of the manuscript and Mohamed Amigh for staining the sections by H&E and Giemsa. This work was funded by Cooperative Research Centres (CRC) for Beef Genetic Technologies, Australia.

Local immune response against larvae of the cattle tick (*Rhipicephalus (Boophilus) microplus)* in Santa Gertrudis cattle with low and high levels of tick resistance

Abstract

Aims

This study investigated the local immune response at larval attachment sites in Santa Gertrudis cattle with low and high levels of tick resistance.

Methods and results

Skin samples with tick larvae attached were collected from Santa Gertrudis cattle at the end of a period of 25 weekly infestations, when the animals manifested highly divergent tick-resistant phenotypes. There was a tendency for more $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, γδ T-cells and neutrophils to concentrate at larval tick attachment site in susceptible cattle than in resistant cattle but the differences were significant only for γδ T-cells and $CD4^+$ cells. Most of the cattle developed intra-epidermal vesicles at the larval attachment site but the predominant cell within or around the vesicles was the neutrophil in susceptible animals and eosinophil in the resistant animals. The monoclonal antibodies (mAbs) specific for CD45 and CD45 RO antigens reacted with skin leukocytes from a higher number of susceptible cattle than resistant cattle.

Conclusion

Our data suggest that some of the cellular responses mounted at larval attachment site are not involved in tick protection. The mAbs specific for CD45 and CD45 RO directly, or a test for CD45 genotype might be developed as markers of tick susceptibility or resistance.

Key Words: *Rhipicephalus microplus*; immune response; cattle; Santa Gertrudis; immuno-fluorescence; leukocytes; skin;

Introduction

Cattle tick, *Rhipicephalus (Boophilus) microplus* is a species complex which currently includes several geographic clades including *R. microplus* in the Australasian region (1, 2). Although the Australasian *R. microplus* clade is now known as the subspecies *R. australis*, the remainder of this article will refer to it as *R. microplus* or 'cattle tick'. *R.microplus* is an economically important tick for the cattle industry worldwide causing in the vicinity of $US22-30b in losses per annum (3). In addition to the direct effects of feeding on blood, hypersensitivity reactions and damage to the hide, *R. microplus* is vector for significant pathogens including *Babesia* spp and *Anaplasma* spp. Infestations with this tick have been commonly controlled through frequent application of chemical acaricides and management (4). Widespread development of acaricide resistance, public concern with worker, environmental and food safety and the increasing costs associated with discovery of new acaricides stimulated interest in alternative methods to control *R. microplus,* including vaccination (5, 6). A thorough understanding of the molecular mechanisms underlying the tick-host relationship and the protective immune response mounted by the host will help design effective vaccines against the *R. microplus* species group.

Bos indicus breeds are less susceptible to infestation with *R. microplus* than *B. taurus* breeds and develop a more effective resistance (7, 8). However, cattle from both species manifest considerable variation in resistance to *R. microplus* (8). In Australia, increasing pressure from domestic and overseas consumer markets is driving producers to introduce more *B. taurus* genetic content into their herds due to the European breeds' superior productivity and meat quality (9). Composite breed animals such as the Santa Gertrudis (5/8 *B. taurus* and 3/8 *B. indicus*), present an attractive alternative to pure *B. indicus* cattle in tick-endemic regions of northern Australia due to their blend of good meat quality and reproductive traits, together with the ability to acquire high levels of tick-resistance (8). The resistance to *R. microplus* is heritable but the mechanisms of resistance in both *B. indicus* and *B. taurus* cattle are not well understood despite intensive research (10). Resistant cattle impair the ability of ticks to attach and feed, resulting in a reduction of the proportion of female ticks that mature, a reduction in the weight of the engorged females and the number and the viability of the eggs laid by female ticks (11-13). Resistance to infestation is directed against all tick stages but it is manifested

primarily against the larval stage in the first 24 h of their parasitic life. In both *B. indicus* and *B. taurus* cattle with high levels of resistance, up to 90% of the larvae are lost within 24 h after infestation (11, 14).

The mechanisms of protection and possible explanations for the differences in resistance to tick infestation between *B. indicus* and *B. taurus* include variation in the structure and physiology of the skin (15, 16), the density of arteriovenous anastomoses at the skin surface (17), histamine concentrations at the larval attachment site (18), self-grooming stimulated by and directed to larval stages (12), and histological features of the tongue (19). However, the immune response plays an important role in protection (10, 20). In cattle with natural infestations the contribution to host resistance of circulating IgG and IgM specific to tick antigens (Ag) is debatable (9, 21) but there is evidence that the cellular immune response is essential for tick resistance (10, 20, 22, 23). Resistance in *B. taurus* cattle was associated with a Type I hypersensitivity reaction to larval allergens and it was correlated with eosinophil concentration and degree of degranulation, mast cell disruption and number of intra-epidermal vesicles at the larval attachment site (20, 23, 24).

There are differences in the local immune response mounted against larvae of *R. microplus* by *B. taurus* and *B. indicus* cattle (7, 25-27). In the early stages of the infestation, infiltrations with neutrophils predominate at larval attachment sites in *B. taurus* cattle, whereas infiltrations with T-cells predominate at larval attachment sites in *B. indicus* cattle (7, 25). Under similar experimental conditions more $CD25^+$, γδ T-cells concentrated at the larval attachment sites in *B. indicus* cattle that developed high resistance to tick infestations than in *B. taurus* cattle that developed only low or moderate tick resistance, which suggested a protective role for these two cell phenotypes (25). There was a tendency for the density of $CD3^+$, $CD4^+$, $CD8^+$, cells to be higher in *B. indicus* cattle than in *B. taurus* cattle. Later in the infestation, massive infiltrations with neutrophils and development of intra-epidermal vesicles filled with neutrophils at larval attachment sites in *B. indicus* cattle, together with massive infiltrations of T cells, suggested a role for neutrophils in tick rejection, apparently in contrast with the early response to infestation, in which neutrophils were prominent at the attachment sites in susceptible cattle (10).

The research on immune response mounted by composite breeds (Santa Gertrudis) against *R. microplus* is scarce. In Santa Gertrudis cattle infested with *R. microplus* there was no association of any peripheral blood leukocyte phenotype ($CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD25^+$, γδ T-cells, MHC class II antigen cells and WC3 cells) with resistance or susceptibility to tick infestation (9). As such there are no reports to phenotype and quantify the leukocyte populations infiltrating the larval attachment sites in composite breeds with different levels of tick resistance. Our aim was to compare the leukocyte subpopulations infiltrating the area around mouthparts of larvae of *R. microplus,* and therefore potentially involved in tick rejection, in resistant and susceptible Santa Gertrudis cattle and to identify cell phenotypes that might be associated with resistance in this composite breed.

Materials and methods

Animals

The trial was conducted with the approval of the University of Queensland Animal Ethics Committee for Production and Companion animals (Approval number: SVS/864/06/CRC and SVS/872/07/CRC). Thirty-five Santa Gertrudis heifers aged 12 months, sourced from a tick-free area of Australia and therefore naïve to *R. microplus* were used in these trials. All animals had been vaccinated against *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*, prior to the commencement of the trial, which took place in animal facilities near Brisbane (Pinjarra Hills, latitude 27.5° and longitude 152.9°), Queensland, Australia (the infested animals were kept in separate facilities from uninfested control animals). Thirty cattle were infested weekly for 25 weeks with 10,000 (0.5 g) larvae (see section below 'ticks') of *R. microplus* that were applied to the neck and withers (9). The infestations occurred in two episodes: there were 13 initial, weekly infestations through winter from May through to July and then, after a one month break, there were 12 further weekly infestations from September through November. In addition to the artificial infestations, the cattle were exposed to ticks under natural conditions in the tick-infested pastures. Five cattle were not infested with ticks and were kept in areas that were ascertained to be free of ticks, and served as tick-free control animals. To prevent infestation

of the un-infested, control cattle, the infested and control animals were kept in different locations (6 km apart) but under similar conditions. The level of host resistance to *R. microplus* was measured by counting the semi-engorged female ticks on day 21 following each larval infestation using the standard method (8). Six animals that were consistently identified as the most resistant animals during the trial were classified as 'Resistant', six animals consistently identified as being the least resistant animals during the same time period were classified as 'Susceptible,' and the rest were classified as 'Middle' (18 animals). The final tick count suggested that on 'Resistant' cattle only 1.1% of the applied ticks matured (high resistant animals according to Utech et al., 1978) while on the 'susceptible' animals 12% of the applied ticks matured (very low resistant animals according to Utech et a., 1978) (9). Because by the end of the study, the count of standard ticks included those arising from natural infestation, our results would be expected to underestimate the mortality of ticks and hence underestimate the host resistance of cattle. This was not considered to be a problem with respect to relative ranking of animals within the trial. Samples from 'Susceptible', 'Resistant' and uninfested animals were used in the present paper.

Ticks

The ticks used in this study were *R. microplus* of the Non-Resistant Field strain (NRFS) (28) that was maintained free of *Babesia* and *Anaplasma* organisms at the Queensland Department of Agriculture and Fisheries' Biosecurity Science Laboratories. Larvae were maintained at 28°C and approximately 95% humidity and applied to animals 7-14 days after hatching. Ticks were applied to the cattle in this study by carefully shaking over the dorsum of cattle, while cattle were restrained in a crush.

Collection and processing of skin samples

Tissue samples with tick larvae attached and feeding in the skin were collected from the perineal area of the cattle within 24 hours after infestation with 10,000 larvae of *R. microplus*. The cattle were restrained in a crush and given an epidural injection of 5 mL of lignocaine 20 mg/mL (Troy Laboratories Pty. Limited, Sydney, Australia) to desensitise the perineum. Skin biopsies were collected with 8 mm biopsy punches (Paramount Surgimed Ltd., New Delhi, India) and within 10 min

of collection were placed in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan.) that was frozen in isopentane (Labscan Asia Co., Ltd., Bangkok, Thailand) cooled with liquid nitrogen. Skin samples were similarly collected and processed from the perineum area of uninfested cattle.

Immuno-fluorescence labeling of cells

The phenotypes of the cells present in the skin of the cattle were identified by double immuno-fluorescence labeling using the antibodies shown in table 1. Briefly, 6 µm thick cryosections were mounted on PolysineTM glass slides (Menzel-GmbH & Co KG, Braunschweig, Germany) and dried overnight at room temperature (RT) with a fan. Next the sections were fixed in cold ethanol (4 ºC) for 8 min. Following fixation the background staining was blocked with Image-iT FX signal enhancer (Invitrogen, Carlsbad, California, USA) followed by 10% [v/v] goat serum in 1% [w/v] bovine serum albumin (BSA, Sigma, St Louis, USA), in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.4 mM KH_2PO_4). The cryosections were further incubated overnight at 4 ºC in a humidified chamber with monoclonal antibodies (100 µL per section) for specific leukocyte receptors (Table 1) diluted in 1% [w/v] BSA/PBS. IgG1, IgG2a and IgM negative control mouse monoclonal antibodies (DakoCytomation, Carpinteria, California, USA) in similar concentrations to the receptor specific antibodies were used as negative controls. The cryosections were washed in PBS and incubated for 40 min at RT with goat anti-mouse isotype-specific antibodies (100 µL per section) conjugated with fluorescein isothiocyanate (FITC) or Texas Red (Invitrogen, Carlsbad, California, USA) and diluted 1/400 [v/v] in 1% [w/v] BSA/PBS. After washing with PBS the nuclei were stained with DAPI dilactate (100 µL per section) (Invitrogen, Carlsbad, California, USA) and the slides were mounted with mounting medium (KPL, Gaithersburg, Maryland, USA). The slides were examined and photographed using an epifluorescent microscope, Olympus BX 51 (Olympus, Tokyo, Japan), equipped with a digital camera (Model DP 70, Olympus, Tokyo, Japan). The images to be published were imported into Microsoft Office Picture Manager and the contrast/brightness adjusted similarly for all.

Enumeration of the cells

Enumeration of the cells was done as previously described (Constantinoiu et al., 2010). Briefly, the cells were counted in one slide (one tick attachment site) for each animal from the tick-infested groups. For all cell subpopulations except MHC class II-expressing cells, the labelled cells were manually counted in an area of 1.05 mm^2 (12 adjacent, non-overlapping high power microscopic fields (40 × objective), three on each side of the tick mouthparts (1 mm from mouthparts in each direction) and two deep from the epidermis (0.5 mm deep in the skin from the level of superficial epidermis)) with image analysis software (NIS-Elements Advanced Research, Nikon, Japan). Cells were counted by a technician blinded to the group of cattle and infestation status of the samples. The pattern of staining by MHC class II antigen specific antibody did not allow us to count individual cells as reliably as other cell types because not all cells were well defined and there was some overlapping of cells. Cells were similarly counted in 12 microscopic fields in the skin of each of the five un-infested cattle.

Histological staining of the sections

Cryosections cut and dried overnight as described above were fixed in 10% Neutral Buffered Formalin (NBF) for 10 minutes at RT, washed three times in distilled water and stained by Haematoxilin & Eosin (H&E) and Giemsa. The eosinophils infiltrating the areas around the tick mouthparts were counted as described above. The epidermis and dermis were assessed for cellular, vascular and structural changes as previously described (16). Each of 15 parameters (Table 4) was scored on a scale of 0–5 as follows: 0 = within normal limits; 1 = minimal change; 2 = mild change; 3 = moderate change; 4 = severe, focal change; 5 = severe, extensive change.

Statistical Methods

The counts of cell numbers on the skin were analysed using a generalised linear model (McCullagh and Nelder 1989) under a Poisson distribution with the logarithm link function, using GenStat (2016). The dispersion parameter was estimated and adopted for the residual, because the data tended to be

over-dispersed. Protected pairwise testing was used to test differences between the treatment group means. The probability level of 0.05 (5%) was used for all significance tests.

Results

1) Reactivity of the antibodies with cells from the skin of the cattle

The mAb specific for CD45 antigen (leukocyte common antigen) labeled cells in the skin of only one animal among the six tick-resistant cattle (17%). However, the same antibody labeled cells in the skin of three out of the six susceptible cattle (50%). The antibody specific for CD45RO antigen (activated cells, memory T cells) reacted with cells from the skin of two out of the six tick resistant cattle (33%) and with cells of five out of the 6 susceptible cattle (83%) (Table 2). For the CD45 and CD45RO specific mAbs combined, the leukocytes of 67% of the susceptible cattle showed antibody reactivity, vs. 25% for the resistant cattle. This difference was significant ($P = 0.041$). These two mAbs were not probed with sections cut from the skin of naïve cattle and the observations described here included the two samples that were later excluded because ticks were assessed as having fed for more than 24 h. No obvious differences regarding the pattern or intensity of staining among the animals in this trial were observed for any of the other mAbs used in this trial (Table 1).

2) Populations of the various cell types in the skin of resistant, susceptible and uninfested cattle

The cells infiltrating an area around tick attachments were counted in 5 resistant animals and 5 susceptible animals only because in two cattle the skin samples collected contained ticks that were evidently attached and had been feeding in the skin for more than 24 h (Fig. 10). Data showing the counts of the cell subpopulations in the three groups of cattle are presented in Fig. 1 and example micrographs are presented in Fig. 2-9.

Both susceptible and resistant cattle had significantly more T cells ($CD3^+$ receptor) ($P<0.05$) in the skin, at tick attachment sites than were counted in skin biopsies of the control, uninfested cattle. The number of T cells around the tick mouthparts was higher in susceptible cattle than in resistant cattle

but the differences were not significant ($P<0.05$) (Fig. 1a). The number of γδ T cells and $CD4^+$ cells in the skin of infested animals (at tick attachment sites) from both groups (susceptible and resistant) of cattle was higher than in the skin of the cattle from the control group. For both γδ T cells and $CD4^+$ cells the numbers in susceptible animals were significantly higher ($P<0.05$) than the number of cells in resistant and naive animals (Fig. 1b & c). The number of $CD8^+$ cells at tick attachment sites in the skin of infested cattle from both groups was significantly higher ($P<0.05$) than in the skin of the control, uninfested, cattle. The number of $CD8^+$ cells in the skin of susceptible cattle was similar to that in the skin of resistant cattle (Fig. 1d).

The number of $CD25^+$ cells at tick attachment site was significantly higher ($P<0.05$) only in infested, susceptible animals than uninfested controls (Fig. 1e). Extremely few B cells (less than one cell/field) were counted in the skin of animals from all groups. The number of neutrophils at tick attachment sites was significantly higher ($P<0.05$) in the infested animals from both groups than in the naïve animals, in which they were extremely rare (less than four cells per field and in 75% of fields zero cells). The number of neutrophils in the skin of susceptible cattle was similar to that in the skin of the resistant cattle ($P>0.05$) (Fig. 1f). The number of eosinophils at tick attachment sites in the skin of infested cattle from both groups (resistant and susceptible) was significantly higher ($P<0.05$) than in the skin of naïve animals. The numbers of eosinophils in the skin of resistant animals were similar to those in the skin of susceptible animals ($P<0.05$) (Fig. 1g).

MHC class II-expressing cells could not be reliably quantified because the shape of the cells was not well defined and very often the cells overlapped. Infiltrations with MHC class II-expressing cells were apparent at the tick attachment sites of susceptible (Fig. 4c) and resistant cattle (Fig. 3c & 6d) and no obvious differences were observed between susceptible and resistant cattle.

3) Type of reaction at tick attachment site

The skin reaction at tick attachment sites varied from none (Fig. 2) to small, empty intra-epidermal vesicles with or without visible infiltrations in the adjacent skin (Fig. 3 & 4) and large epidermal vesicles filled mainly with neutrophils (Fig. 5) or eosinophils (Fig 6) or both types of cells. In both

resistant and susceptible animals most of the animals had intra-epidermal vesicles at the tick attachment site (66% in susceptible cattle and 80% in resistant cattle) but the predominant type of cell within or around the vesicles was the neutrophil in susceptible animals and the eosinophil in the resistant animals (Table 3, Fig. 5 and Fig. 6). MHC class II antigen cells consistently infiltrated the areas around vesicles in both susceptible and resistant cattle but could only be found in extremely small numbers, if any, within the intra-epidermal vesicles (Fig. 3c, 4c, 5d and 6d). Neutrophils (Fig. 4b), eosinophils (Fig. 3a) and MHC class II-expressing cells (Fig. 3c and 4c) infiltrated the areas closest to the tick mouth parts. In resistant animals neutrophils apparently did not infiltrate or accumulate in the tick feeding areas (Fig. 3b and 6b). In susceptible animals neutrophils infiltrated all tick attachment sites (Fig. 4b) but one (Fig. 2a). Furthermore, massive infiltrations with neutrophils that appeared as continuous bands in the dermis were seen in two susceptible cattle (Fig. 7). Tissue lysis around clusters of neutrophils was observed in the epidermis of two susceptible cattle, suggesting that these cells are involved in the formation of vesicles (Fig. 4b, 8). Infiltrations with MHCII cells around tick attachment sites or vesicles were seen in most animals from both groups (Fig. 3c, 4c and 6c).

4) Degree of inflammatory reaction in the skin

Microscopic comparisons of standard, H&E stained skin biopsies from resistant and susceptible cattle to *R. microplus* had similar features (Table 4). Dermal inflammation, primarily consisting of neutrophils, eosinophils, mast cells and plasma cells was noted in both groups and in some animals was extensive and of moderate to marked severity. Intra-epidermal vesicles were noted in both groups, were of varying size and depth within the epithelium, and the larger lesions more frequently were open, the attenuated, superficial tissue having torn secondary to mechanical trauma or because of the nature of the devitalized tissue. The predominant inflammatory cell type within the vesicles was split between neutrophilic and eosinophilic. Most often both cell types were present.

Discussion

The mAbs specific for CD45 and CD45 RO antigens reacted with skin leukocytes from a larger number of susceptible cattle than resistant cattle but the differences were not significant for either of the two mAbs individually. However, when the reactivity of the two mAbs was combined the differences in the reaction of these mAbs with skin leukocytes from susceptible and resistant cattle were significant ($P<0.05$). A previous trial using a small number of cattle (three *B. taurus* and three *B. indicus* cattle) found obvious differences in the reactivity of the mAbs specific for CD45 and CD45RO between *B. taurus* and *B. indicus* cattle: both CD45 and CD45RO antibodies reacted with skin leukocytes from *B. taurus* but neither antibody reacted with skin leukocytes from *B. indicus* cattle (29).Thus, the epitopes recognized by these mAbs are likely to occur on leukocytes with CD45 (protein tyrosine phosphatase, receptor type-C, or *PTPRC*) alleles inherited from *B. taurus* cattle while the absence of the epitopes is likely to occur on cells with *PTPRC* alleles inherited from *B. indicus* cattle. It is conceivable therefore that in composite breeds (*Bos indicus* × *Bos taurus*) these antibodies might be used as markers or indicators of tick susceptibility/resistance. Allelic polymorphism in *PTPRC* gene, associated with distinct evolutionary families of cattle has been described (30). The potential value of CD45 and CD45RO antibodies being useful as markers for tick susceptibility/resistance requires testing using larger numbers of animals, molecular genotyping, and possibly more mAbs specific to other epitopes of CD 45/CD45RO antigens.

The cellular reaction at the tick attachment site varied very much, from no reaction (Fig. 2) to large vesicles with massive cellular infiltrations (Fig. 5). Substantial variation was noted even among larvae attached at different sites on a single animal. This variation in response shows clearly that the reaction to the attachment and feeding of ticks is dynamic and changes occur rapidly. The larvae of *R. microplus* attempt approximately five attachments in the first 24 hours of their parasitic life (31) and whether an attachment site is a successful, final attempt or an early, unsuccessful attempt might also influence the cellular infiltrations that we observed at larval attachment sites. Our sampling method (dependent on a tick being present and attached) precludes the examination of a site where an attempt to feed was unsuccessful and the tick had moved to try to feed elsewhere. Development of intra-epidermal vesicles represents a quick (they form within 3-5 hours after larval attachment) and common host reaction at the larval attachment site (10, 20, 25) (Figs. 3-6) and it was generally

associated with development of resistance in both *B. indicus* and *B. taurus* cattle as the larvae can no longer anchor and/or feed in the skin of the host and detach (Fig. 9) (10, 20). Our limited data show that intra-epidermal vesicles do not develop at the attachment sites of ticks that had successfully attached in the skin for longer than 24 hours although huge cellular infiltrations are sometimes present in the skin beneath the attachment (Fig. 10). Previous research revealed higher incidence of epidermal vesicles in the skin of *B. taurus* resistant animals than in the skin of *B. taurus* susceptible cattle (20), which concurs with our data. However, in the previous study it was found that only eosinophils infiltrated the epidermis and caused the intra-epidermal vesicles (20). In contrast we have found that both eosinophils and neutrophils infiltrated the epidermis and in intra-epidermal vesicles there was a tendency for eosinophils to be the dominant cell type in the resistant animals and neutrophils in the susceptible animals. Furthermore, our data show that neutrophils are involved in lysis of epidermis and formation of the intra-epidermal vesicles in susceptible cattle, consistent with research undertaken on *R. sanguineus* in dogs (32). Formation of eosinophilic vesicles occurs more quickly in resistant *B. taurus* cattle than in susceptible *B. taurus* cattle (20) and eosinophils might be more effective than neutrophils in tick protection. Ingested eosinophils seem to have a deleterious effect on the gut of ticks (33) but ingested neutrophils seem not to have a damaging effect on ticks as larvae of *R. microplus* can feed on them without apparently being affected (25). On the other hand neutrophil-filled intra-epidermal vesicles that prevented larvae from anchoring in the skin were observed in highly resistant *B. indicus* cattle (10).

Many elements of the immune response, including dendritic cells, T and B-cells, NK cells, macrophages, eosinophils, neutrophils, basophils, mast cells, immunoglobulins, cytokines are involved in the development and expression of resistance to tick infestation (34). However, the particular elements involved depend on many factors, including the species and breed of the host as well as tick species and tick lifecycle stage (34, 35).

In infestations with *R. microplus* the mechanisms of resistance are primarily manifest against larvae within 24 hours after finding a host and commencement of their parasitic phase (11, 14), which is the reason why larvae were the target of this study. The local immune response mounted at the larval attachment site is important in rejection of this lifecycle stage and tick protection (10, 20). Generally,

high infiltrations with particular leukocyte phenotypes at larval attachment sites were associated with development of an adaptive immune response and likely protection (10, 20, 25). In the present trial, for all the leukocyte phenotypes investigated, more cells were counted at larval attachment sites in infested animals from both groups of cattle than in the skin of control animals (Fig. 1), which is equally consistent with the development of an adaptive immune response and a pathological, non-protective response. However, except for eosinophils, there was a tendency for more cells from all the cell phenotypes investigated ($CD3^+$, γδ T cells, $CD4^+$, $CD8^+$, $CD25^+$ cells and neutrophils) to be higher at larval attachment sites in susceptible cattle. This suggested that most of the cellular responses represent pathology rather than effective defense. Furthermore, while the apparent differences between naïve and susceptible cattle were significant ($P<0.05$) for all phenotypes investigated, the differences between naïve and resistant cattle were not significant ($P>0.05$) for γδ T cells, $CD4^+$ cells and $CD25^+$ cells. This contrasts with our previous study, in which resistant cattle (*B. indicus*) concentrated more $CD3^+$, $CD4^+$, $CD8^+$ cells and neutrophils and significantly more γδ T cells and $CD25^+$ at the larval attachment site than the low/moderate resistant cattle (*B. taurus*) (25). As a result, it was suggested that γδ T cells and $CD25^+$ were important in cattle tick protection, $CD25^+$ cells possibly through regulation of the intensity of the local effector responses and γδ T-cells through their role in integrating the innate and adaptive immune responses and wound healing (36-38). $CD4^+$ cells might be important for tick resistance, through their role in polarization of the immune response to a Th2 profile and regulation of the intensity of cell infiltrations, especially neutrophils and eosinophils, in the skin at tick attachment sites via the cytokines they secrete (39). The differences in (the size of) cellular infiltrations at larval attachment site between resistant and susceptible cattle are supported by the counts of peripheral $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$ and γδ T cells in the same animals: the numbers of cells from these phenotypes were similar or slightly higher in susceptible animals (9). In the current trial only the eosinophils were found in higher numbers at larval attachment sites in resistant cattle but the differences between resistant and susceptible cattle were not significant. In a previous trial, eosinophils infiltrated the larval attachment sites earliest after larvae successfully attached to the skin of the host and they were more numerous in *B. taurus* than in *B. indicus* cattle

and in follow-up infestations compared with primary infestations (27). Furthermore, in *B. taurus* cattle the level of resistance to tick infestation correlated with eosinophil concentration and degranulation at larval attachment sites (20, 23). In our trial the number of eosinophils at larval attachment sites tended to be higher in resistant animals than in susceptible animals but it was not significantly higher. The difference between the results of Schleger et al (1976) and our results might be explained by the differences in the size of the area over which the eosinophils were counted at larval attachment sites, being smaller and located immediately under the larval mouthparts in study of Schleger et al (1976) versus larger and located around the tick mouthparts in our trial, the time of collection of skin samples (3 h post infestation in study of Schleger et al (1976 and 24 h post infestation in the present trial) and the genetic composition of the cattle (*B. taurus* in study of Schleger et al (1976) and a composite breed, Santa Gertrudis: 5/8 *B. taurus* and 3/8 *B. indicus,* in the present trial). Taken together these results provide some support for the view that in *B. taurus* cattle eosinophil concentration at larval attachment sites is associated with larval rejection (20, 23).

Tick saliva has proven immunomodulatory effects and can cause local immunosupression that helps the tick survive and feed on the host (39, 40). Salivary extracts from females of *Dermacentor andersoni* and *Ixodes scapularis* downregulated the expression of the adhesion molecules ICAM-1, VCAM-1 and P-selectin on the endothelial cells that is likely to interfere with leukocyte extravasation from the blood vessels and their migration to the tick attachment site (41). *R. microplus* can also modulate the expression of adhesion molecules (ICAM-1, VCAM-1, P-selectin and E-selectin) at adult tick attachment site but the effect at larval attachment site was not described (42). The immuno-suppressive effects of saliva of *R. microplus* on certain components of the immune response are more intense in susceptible breeds of cattle than in resistant ones (42). The susceptible cattle in the present trial concentrated more leukocytes at the larval attachment site than the resistant cattle and two of them had huge infiltrations with neutrophils that formed continuous bands in the skin. This suggests that recruitment of leukocytes to the larval attachment site is not impaired in susceptible cattle any more than in the resistant cattle. This concurs with Piper et al (2009), who found that expression of genes coding for cytokines and complement factors with chemotactic properties (CXCL-8, CXCL-2, CXCL-5, CCL-2, CCL-8 and regakine-1) at the larval attachments sites was higher in tick-susceptible

cattle. The differences in the amount and composition of saliva secreted by larvae within 24 hours of their parasitic life and female ticks might explain the differences between research of Carvalho et al (2010) and the results of Piper et al (2009) and those of the present trial (43). This suggests that larvae might lack the protection afforded by the immunosuppressive effects of saliva to adult female ticks and are more susceptible to host rejection.

B. indicus cattle have a long evolutionary association with *R. microplus* and it was suggested that this has resulted in an adaptive tolerance manifested by reduced inflammatory cellular reaction at tick attachment site (16, 27), which might explain the low cellular infiltrations in resistant animals in the present trial. This is consistent with our hypothesis that some of the results from the earlier study would have been a consequence of indicine v taurine difference, independent of the protective immune response mounted to tick infestation. It also suggests that *R. microplus* larvae do not impair the recruitment of cells to larval attachment sites but they affect the responsiveness and the polarization of the immune response towards a Th1 or Th2 response (39). Alternatively, the timing of sample collection (seven infestations carried out over two months in Constantinoiu et al. (2010) vs twenty-five artificial infestations carried out over more than seven months in the present trial) and the obviously higher antigenic stimulation of the susceptible animals than that of resistant cattle (generally 6 times more ticks matured on the body of susceptible cattle) might have affected the magnitude and composition of cellular infiltrations in the skin of the cattle in general and at the larval attachment site in particular.

References

1. Burger TD, Shao R, Barker SC. Phylogenetic analysis of mitochondrial genome sequences indicates that the cattle tick, Rhipicephalus (Boophilus) microplus, contains a cryptic species. Mol Phylogenet Evol. 2014;76:241-53.
2. Low VL, Tay ST, Kho KL, Koh FX, Tan TK, Lim YA, et al. Molecular characterisation of the tick Rhipicephalus microplus in Malaysia: new insights into the cryptic diversity and distinct genetic assemblages throughout the world. Parasit Vectors. 2015;8:341.
3. Lew-Tabor AE, Rodriguez Valle M. A review of reverse vaccinology approaches for the development of vaccines against ticks and tick borne diseases. Ticks Tick Borne Dis. 2016;7(4):573-85.
4. Jonsson NN. Control of cattle ticks (Boophilus microplus) on Queensland dairy farms. Aust Vet J. 1997;75(11):802-7.
5. Guerrero FD, Miller RJ, Perez de Leon AA. Cattle tick vaccines: many candidate antigens, but will a commercially viable product emerge? Int J Parasitol. 2012;42(5):421-7.
6. George JE, Pound JM, Davey RB. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. Parasitology. 2004;129 Suppl:S353-66.
7. Piper EK, Jackson LA, Bagnall NH, Kongsuwan KK, Lew AE, Jonsson NN. Gene expression in the skin of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Vet Immunol Immunopathol. 2008;126(1-2):110-9.
8. Utech KB, Wharton RH, Kerr JD. Resistance to Boophils microplus (Canestrini) in different breeds of cattle. Aust J Agric Res. 1978;29:885-95.
9. Piper EK, Jonsson NN, Gondro C, Vance ME, Lew-Tabor A, Jackson LA. Peripheral cellular and humoral responses to infestation with the cattle tick Rhipicephalus microplus in Santa Gertrudis cattle. Parasite Immunol. 2017;39(1).
10. Jonsson NN, Piper EK, Constantinoiu CC. Host resistance in cattle to infestation with the cattle tick Rhipicephalus microplus. Parasite Immunol. 2014;36(11):553-9.
11. Roberts JA. Resistance of cattle to the tick boophilus microplus (canestrini). II. Stages of the life cycle of the parasite against which resistance is manifest. J Parasitol. 1968;54(4):667-73.
12. Koudstaal D, Kemp DH, Kerr JD. Boophilus microplus: rejection of larvae from British breed cattle. Parasitology. 1978;76(3):379-86.
13. Willadsen P. Immunity to ticks. Adv Parasitol. 1980;18:293-311.
14. Wagland BM. Host resistance to cattle tick (Boophilus microplus) in Brahman (Bos indicus) cattle. IV Ages of ticks rejected. Aust J Agric Res. 1979;30:211-8.
15. Kongsuwan K, Josh P, Colgrave ML, Bagnall NH, Gough J, Burns B, et al. Activation of several key components of the epidermal differentiation pathway in cattle following infestation with the cattle tick, Rhipicephalus (Boophilus) microplus. Int J Parasitol. 2010;40(4):499-507.
16. Piper EK, Jackson LA, Bielefeldt-Ohmann H, Gondro C, Lew-Tabor AE, Jonsson NN. Tick-susceptible Bos taurus cattle display an increased cellular response at the site of larval Rhipicephalus (Boophilus)microplus attachment, compared with tick-resistant Bos indicus cattle. Int J Parasitol. 2009.
17. Schleger AV, Lincoln DT, Bourne AS. Arteriovenous anastomoses in the dermal vasculature of the skin of Bos taurus cattle, and their relationship with resistance to the tick, Boophilus microplus. Aust J Biol Sci. 1981;34(1):27-35.
18. Kemp DH, Bourne A. Boophilus microplus: the effect of histamine on the attachment of cattle-tick larvae--studies in vivo and in vitro. Parasitology. 1980;80(3):487-96.
19. Verissimo CJ, D'Agostino SM, Pessoa FF, de Toledo LM, Santos IK. Length and density of filiform tongue papillae: differences between tick-susceptible and resistant cattle may affect tick loads. Parasit Vectors. 2015;8:594.

20. Schleger AV, Lincoln DT, McKenna RV, Kemp DH, Roberts JA. Boophilus microplus: cellular responses to larval attachment and their relationship to host resistance. Aust J Biol Sci. 1976;29(5-6):499-512.
21. Kashino SS, Resende J, Sacco AM, Rocha C, Proenca L, Carvalho WA, et al. Boophilus microplus: the pattern of bovine immunoglobulin isotype responses to high and low tick infestations. Exp Parasitol. 2005;110(1):12-21.
22. Riek RF. Factors influencing the susceptibility of cattle to tick infestation. Aust Vet J. 1956;32:204-8.
23. Schleger AV, Lincoln DT, Kemp DH. A putative role for eosinophils in tick rejection. Experientia. 1981;37(1):49-50.
24. Willadsen P, Williams PG, Roberts JA, Kerr JD. Responses of cattle to allergens from Boophilus microplus. Int J Parasitol. 1978;8(2):89-95.
25. Constantinoiu CC, Jackson LA, Jorgensen WK, Lew-Tabor AE, Piper EK, Mayer DG, et al. Local immune response against larvae of Rhipicephalus (Boophilus) microplus in Bos taurus indicus and Bos taurus taurus cattle. Int J Parasitol. 2010;40(7):865-75.
26. Tatchell RJ, Moorhouse DE. The feeding processes of the cattle tick Boophilus microplus (Canestrini). II. The sequence of host-tissue changes. Parasitology. 1968;58(2):441-59.
27. Moorhouse DE, Tatchell RJ. Histological responses of cattle and other ruminants to the recent attachment of ixodid larvae. J Med Entomol. 1969;6(4):419-22.
28. Stewart NP, Callow LL, Duncalfe F. Biological comparisons between a laboratory-maintained and a recently isolated field strain of Boophilus microplus. J Parasitol. 1982;68(4):691-4.
29. Constantinoiu CC, Jonsson NN, Jorgensen WK, Jackson LA, Piper EK, Lew-Tabor AE. Immuno-fluorescence staining patterns of leukocyte subsets in the skin of taurine and indicine cattle. Res Vet Sci. 2013;95(3):854-60.
30. Ballingall KT, Waibochi L, Holmes EC, Woelk CH, MacHugh ND, Lutje V, et al. The CD45 locus in cattle: allelic polymorphism and evidence for exceptional positive natural selection. Immunogenetics. 2001;52(3-4):276-83.
31. Roberts JA. Behavior of larvae of the cattle tick, Boophilus microplus (Canestrini), on cattle of differing degrees of resistance. J Parasitol. 1971;57(3):651-6.
32. Tatchell RJ, Moorhouse DE. Neutrophils: their role in the formation of a tick feeding lesion. Science. 1970;167(920):1002-3.
33. Wikel S. Immunology of the Tick-Host Interface. In: Wikel S, editor. The Immunology of Host-Ectoparasitic Arthropod Relationships. Walingford: CAB INTERNATIONAL; 1996.
34. Brossard M, Wikel SK. Tick immunobiology. Parasitology. 2004;129 Suppl:S161-76.
35. Piper EK, Jonsson NN, Gondro C, Lew-Tabor AE, Moolhuijzen P, Vance ME, et al. Immunological profiles of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Clin Vaccine Immunol. 2009;16(7):1074-86.
36. Born WK, Reardon C, O'Brien RL. The function of γδ T cells in innate immunity. Curr Opin Immunol. 2006;18:31-8.
37. Lahmers KK, Hedges JF, Jutila MA, Deng M, Abrahamsen MS, Brown WC. Comparative gene expression by $WC1^+$ γδ and $CD4^+$ αβ T lymphocytes, which respond to Anaplasma marginale, demonstrates higher expression of chemokines and other myeloid cell-associated genes by $WC1^+$ γδT cells. J Leukoc Biol. 2006;80:939-51.
38. Belkaid Y. Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol. 2007;7(11):875-88.
39. Brake DK, Wikel SK, Tidwell JP, Perez de Leon AA. Rhipicephalus microplus salivary gland molecules induce differential CD86 expression in murine macrophages. Parasit Vectors. 2010;3:103.
40. Wikel S. Ticks and tick-borne pathogens at the cutaneous interface: host defenses, tick countermeasures, and a suitable environment for pathogen establishment. Front Microbiol. 2013;4:337.

41. Maxwell SS, Stoklasek TA, Dash Y, Macaluso KR, Wikel SK. Tick modulation of the in-vitro expression of adhesion molecules by skin-derived endothelial cells. Ann Trop Med Parasitol. 2005;99(7):661-72.
42. Carvalho WA, Franzin AM, Abatepaulo AR, de Oliveira CJ, More DD, da Silva JS, et al. Modulation of cutaneous inflammation induced by ticks in contrasting phenotypes of infestation in bovines. Vet Parasitol. 2010;167(2-4):260-73.
43. Binnington KC. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, Boophilus microplus. Int J Parasitol. 1978;8(2):97-115.
44. Niku M, Ekman A, Pessa-Morikawa T, Iivanainen A. Identification of major cell types in paraffin sections of bovine tissues. BMC Veterinary Research. 2006;2:5.
45. Keresztes G, Takacs L, Vilmos P, Kurucz E, Ando I. Monoclonal antibodies detecting components of the bovine immune system in formaldehyde-fixed paraffin-embedded tissue specimens. Veterinary Immunology and Immunopathology. 1996;52(4):383-92.
46. Bembridge GP, MacHugh ND, McKeever D, Awino E, Sopp P, Collins RA, et al. CD45RO expression on bovine T cells: relation to biological function. Immunology. 1995;86(4):537-44.
47. Davis WC, MacHugh ND, Park YH, Hamilton MJ, Wyatt CR. Identification of a monoclonal antibody reactive with the bovine orthologue of CD3 (BoCD3). Veterinary Immunology and Immunopathology. 1993;39(1-3):85-91.
48. Naessens J, Nthale JM, Muiya P. Biochemical analysis of preliminary clusters in the non-lineage panel. Vet Immunol Immunopathol. 1996;52(4):347-56.
49. Gutierrez M, Forster FI, McConnell SA, Cassidy JP, Pollock JM, Bryson DG. The detection of CD2+, CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in fixed and paraffin embedded bovine tissue using a range of antigen recovery and signal amplification techniques. Veterinary Immunology and Immunopathology. 1999;71(3-4):321-34.
50. Liebana E, Marsh S, Gough J, Nunez A, Vordermeier HM, Whelan A, et al. Distribution and activation of T-lymphocyte subsets in tuberculous bovine lymph-node granulomas. Vet Pathol. 2007;44(3):366-72.
51. Jones M, Cordell JL, Beyers AD, Tse AG, Mason DY. Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies. Journal of Immunology. 1993;150(12):5429-35.
52. Morrison WI, Davis WC. Individual antigens of cattle. Differentiation antigens expressed predominantly on CD4- CD8- T lymphocytes (WC1, WC2). Vet Immunol Immunopathol. 1991;27(1-3):71-6.
53. Taylor BC, Choi KY, Scibienski RJ, Moore PF, Stott JL. Differential expression of bovine MHC class II antigens identified by monoclonal antibodies. Journal of Leukocyte Biology 1993;53(5):479-89.
54. Bensaid A, Hadam M. Individual antigens of cattle. Bovine CD4 (BoCD4). Veterinary immunology and immunopathology. 1991;27(1-3):51-4.
55. Collins RA, Werling D, Duggan SE, Bland AP, Parsons KR, Howard CJ. Gammadelta T cells present antigen to CD4+ alphabeta T cells. J Leukoc Biol. 1998;63(6):707-14.

599 Table 1 Monoclonal antibodies used to characterize cells infiltrating the skin areas around tick mouthparts

Monoclonal antibody designation	Source	Antigen specificity	Isotype	Cellular expression	Dilution used	Reference
CACTB51A	VMRD	CD45	IgG2a	Leukocytes	1/800	(44, 45)
Il-A116	VMRD	CD45RO	IgG3	Activated cells	1/400	(46)
MM1A	VMRD	CD3	IgG1	T cells	1/800	(47)
CH138	VMRD	Neutrophils	IgM	Neutrophils	1/400	(29, 45, 48)
MCA837G	AbD Serotec	CD8	IgG2a	T cytotoxic cells	1/50	(49, 50)
HM57	DakoCytomation	CD79ά	IgG1	B cells	1/100	(51)
IL-A29[a]	ILRI[b]	γδ form of	IgG1	γδ T cells	1/25	(52)

		the T cell receptor					
IL-A21[a]	ILRI[b]	MHC class II antigen	IgG2a	Macrophages, dendritic cells, B cells, activated T cells	1/200	(53)	
IL-A12[a]	ILRI[b]	CD4	IgG2a	T helper cells	1/25	(54)	
IL-A111[a]	ILRI[b]	CD25	IgG1	Activated cells (IL2-R bearing cells)	1/25	(55)	

[a] Monoclonal antibodies from tissue culture supernatant

[b] International Livestock Research Institute, Nairobi, Kenya

Table 2 The reactivity of the antibodies specific for CD45 and CD45RO epitopes with the skin leukocytes of cattle with different levels of tick resistance

Cow tag	CD45	CD45RO
B907-S	+	+
B797-S	+	+
B639-S	+	+
B629-S	-	+
B615-S	-	+
B607-S	-	-
B809-R	+	+
B825-R	-	-
B821-R	-	-
B783-R	-	-
B679-R	-	-
B501-R	-	+/-

Table 3 Type of reaction at tick attachment site in susceptible and resistant cattle

Type of reaction at tick attachment site	Susceptible cattle (6 tick attachments)	Resistant cattle (5 tick attachments)
Absence of any cell infiltration/reaction	1	1
Cellular infiltrations	1 (eosinophils, neutrophils & MHC class II antigen cells)	0
Empty intra-epidermal vesicle with no visible/obvious infiltrations around vesicle	0	1
Empty intra-epidermal vesicle with cellular infiltrations around vesicle	2 (neutrophils & MHC class II antigen cells adjacent to the vesicle)	2 (eosinophils & MHC class II antigen cells adjacent to the vesicle)
Intra-epidermal vesicles filled with cells	1 (neutrophils within vesicle and neutrophils and MHC class II antigen cells adjacent to the vesicle) 1 (neutrophils and eosinophils within vesicle)	1 (eosinophils within vesicles and eosinophils and MHC class II antigen cells adjacent to the vesicle)

641 Table 4 Inflammatory reaction in the skin of the cattle: parameters assessed in the epidermis and dermis and their scores

Type	Naive					Susceptible					Resistant				
Cow tag	B407	B605	B573	B507	B857	B629	B639	B797	B615	B607	B821	B679	B783	B825	B501
Epidermis															
Acanthosis	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Apoptosis/necrosis	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
Acantholysis	0	0	0	0	0	0	0	0	2	2	0	1	3	2	2
Micro-abscess	2	0	0	0	0	2	2	2	2	1	0	2	3	2	2
Subepidermal clefting	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Transepithelial leukocyte migration	2	0	0	0	0	2	3	1	2	2	0	2	3	2	2
Hyperkeratosis - ortho	0	0	0	0	0	2	2	1	1	1	0	1	0	1	1
Hyperkeratosis - para	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermis															
Oedema	0	0	0	0	0	1	2	1	1	2	1	2	2	2	2
Collagen degeneration	0	0	0	0	0	2	2	1	1	1	1	2	2	2	2
Vascular rexn/vasculitis	0	0	0	0	0	1	1	1	1	2	1	1	2	1	1
Transendothelial leukocyte migration	0	0	0	0	0	3	2	1	1	2	1	1	2	1	1
PMN/Eosinophil infiltrate	1	0	0	1	0	3	2	2	2	2	1	2	3	2	2
Mononuclear cell infiltrate	0	0	0	0	0	3	2	2	1	2	1	1	3	1	1

Mast cell infiltrate	0	0	0	0	0	2	2	1	1	2	1	1	2	1	1
Total	**5**	**0**	**0**	**1**	**0**	**23**	**22**	**13**	**16**	**19**	**7**	**16**	**25**	**17**	**17**

Figure legends

Fig. 1 Comparative counts of immune cells at the tick attachment areas in susceptible and resistant cattle. Fig. 1a: T cells, Fig. 1b: γδ T cells, Fig. 1c: $CD4^+$ cells, Fig. 1d: $CD8^+$ cells, Fig. 1e: $CD25^+$ cells, Fig. 1f: neutrophils and Fig. 1g: eosinophils. Means of cells per field for each group of animals and standard error bars are shown. Different letters show significant differences ($P < 0.05$).

Fig. 2. Lack of cellular reaction at larval attachment site in the skin of a susceptible cow. Fig. 2a: H&E staining, Fig. 2b: neutrophils (green), γδ T cells (red) and cell nuclei (blue), Fig. 2c: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; TM: tick mouthparts).

Fig. 3 Empty intra-epidermal vesicle with adjacent eosinophil infiltrations in a resistant cow. Fig. 3a: H&E staining, Fig. 3b: neutrophils (green), γδ T cells (red) and cell nuclei (blue), Fig. 3c: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; V: vesicle).

Fig. 4 Empty intra-epidermal vesicle with neutrophil infiltrations at tick attachment site in a susceptible cow. Fig. 4a: H&E staining, Fig. 4b: neutrophils (green), γδ T cells (red) and cell nuclei (blue), Fig. 4c: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; V: vesicle; TM: tick mouthparts).

Fig. 5 Intra-epidermal vesicle filled mostly with neutrophils at the tick attachment site in a susceptible cow. Fig. 5a: H&E staining, Fig. 5b: neutrophils (green), γδ T cells (red) and cell nuclei (blue), Fig. 5c: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; V: vesicle; TC: tick cement).

Fig. 6 Intra-epidermal vesicle filled mostly with eosinophils at the tick attachment site in a resistant cow. Fig. 6a: H&E staining, Fig. 6b: neutrophils (green), γδ T cells (red) and cell nuclei (blue), Fig. 6c: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; V: vesicle).

Fig. 7 Infiltrations with neutrophils (green) forming a continuous band in the skin of a susceptible cow (E: epidermis; D: dermis).

Fig. 8 Tissue lysis around clusters of neutrophils in the skin of a susceptible cow. Differential interference contrast (DIC) showing the areas of intra-epidermal lysis, neutrophils (green), γδ T cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; FV: forming vesicle).

Fig. 9 Tick fixed in a piece of superficial epidermis that detached from the skin. Fig. 9a: Tick and the superficial epidermis away from the skin spot the tick was fixed (H&E staining), Fig. 9b: The place of skin the tick was initially fixed and the skin damage (H&E staining), Fig. 9c: Tick and the superficial epidermis away from the skin spot the tick was fixed: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue), Fig. 9d: The place of skin the tick was initially fixed: neutrophils (green), γδ T cells (red) and cell nuclei (blue). (E: epidermis; D: dermis; V: vesicle).

Fig. 10 Massive cellular infiltrations at the tick attachment site of a tick fixed in the skin for more than 24 hours. Fig. 10a: H&E staining, Fig. 10b: neutrophils (green), γδ T cells (red) and cell nuclei (blue), Fig. 10c: T cells (green), MHC class II antigen cells (red) and cell nuclei (blue) (E: epidermis; D: dermis; TC: Tick cement; TM: tick mouthparts).

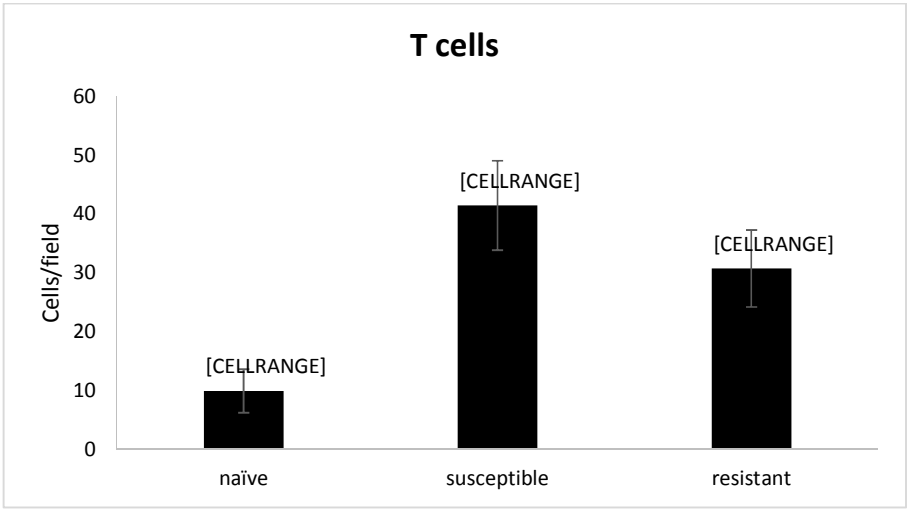
T cells
Cells/field
60
50
40
30
20
10
0
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1a

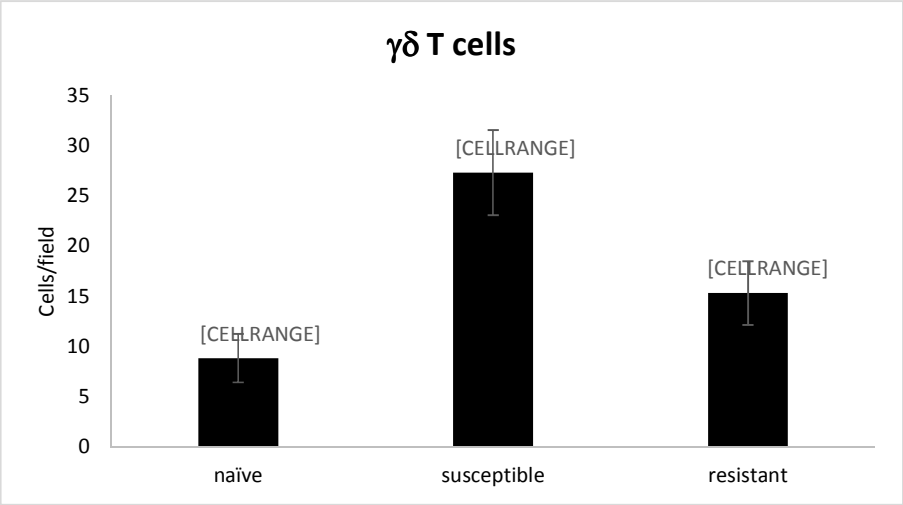
γδ T cells
Cells/field
35
30
25
20
15
10
5
0
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1b

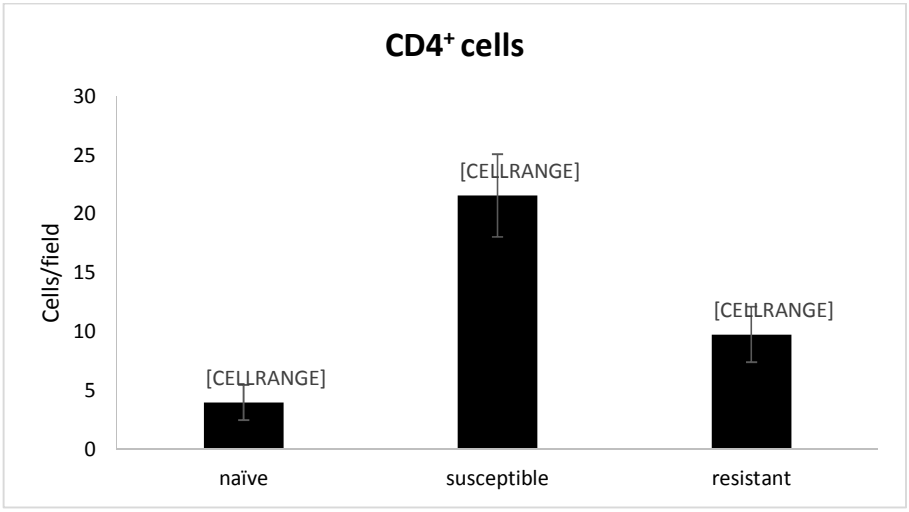
CD4+ cells
Cells/field
30
25
20
15
10
5
0
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1c

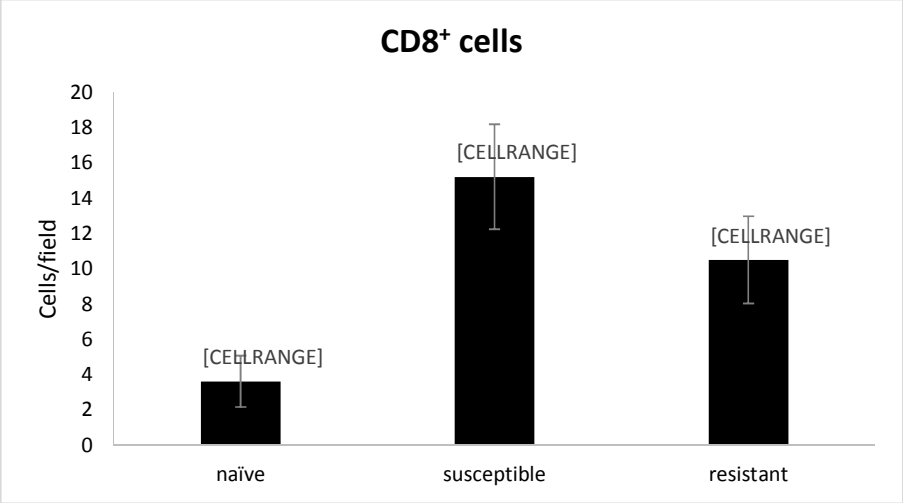
CD8+ cells
Cells/field
20
18
16
14
12
10
8
6
4
2
0
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1d

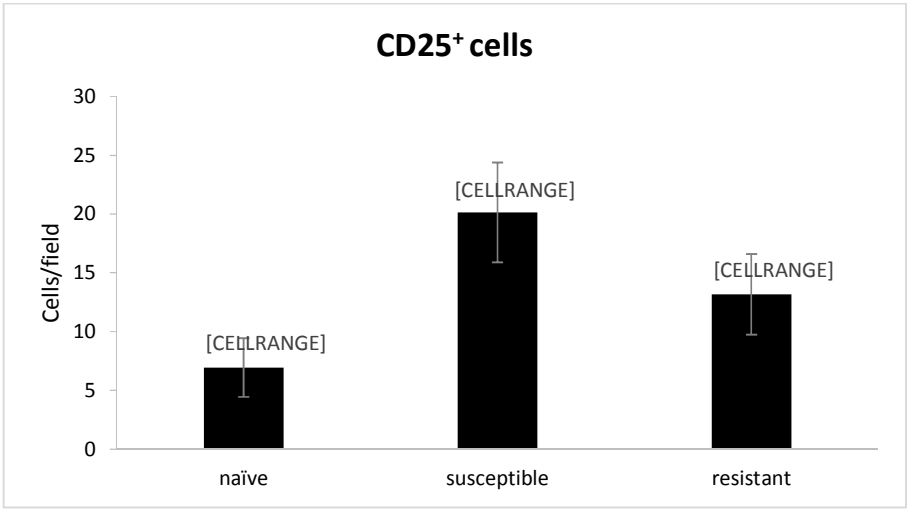
CD25+ cells
Cells/field
30
25
20
15
10
5
0
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1e

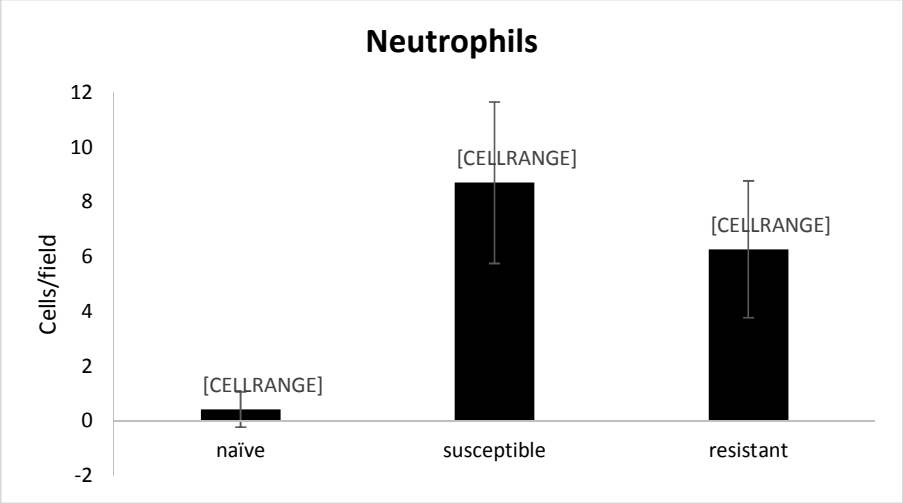
Neutrophils
Cells/field
12
10
8
6
4
2
0
-2
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1f

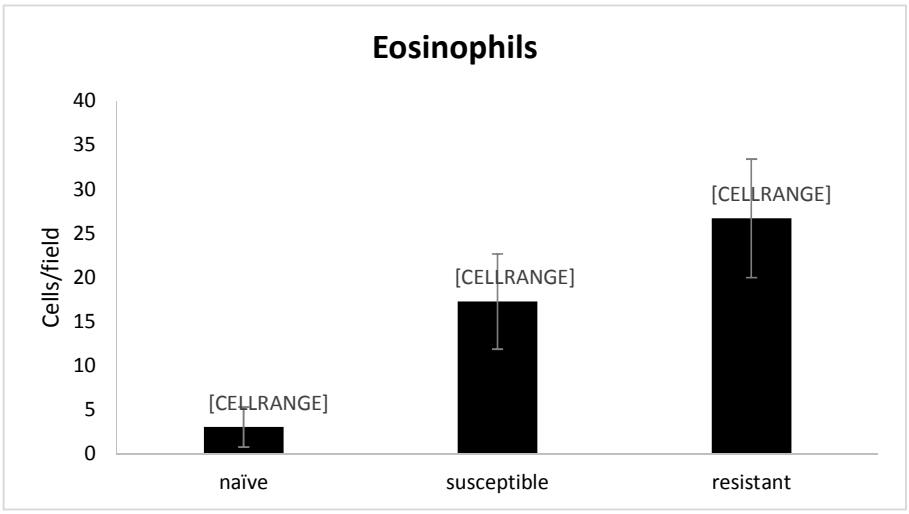
Eosinophils
Cells/field
40
35
30
25
20
15
10
5
0
[CELLRANGE]
[CELLRANGE]
[CELLRANGE]
naïve
susceptible
resistant

Fig. 1g

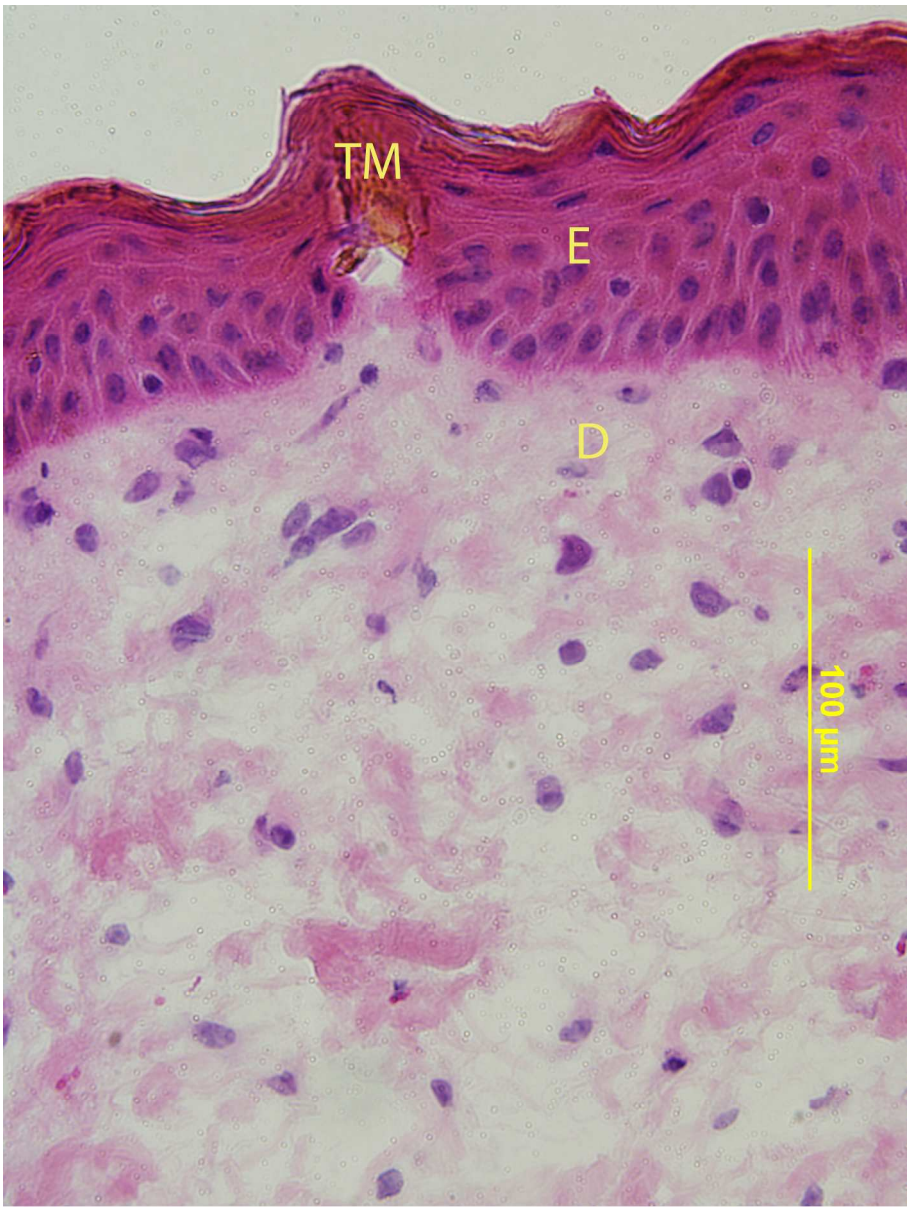

Fig. 2a

239x318mm (300 x 300 DPI)

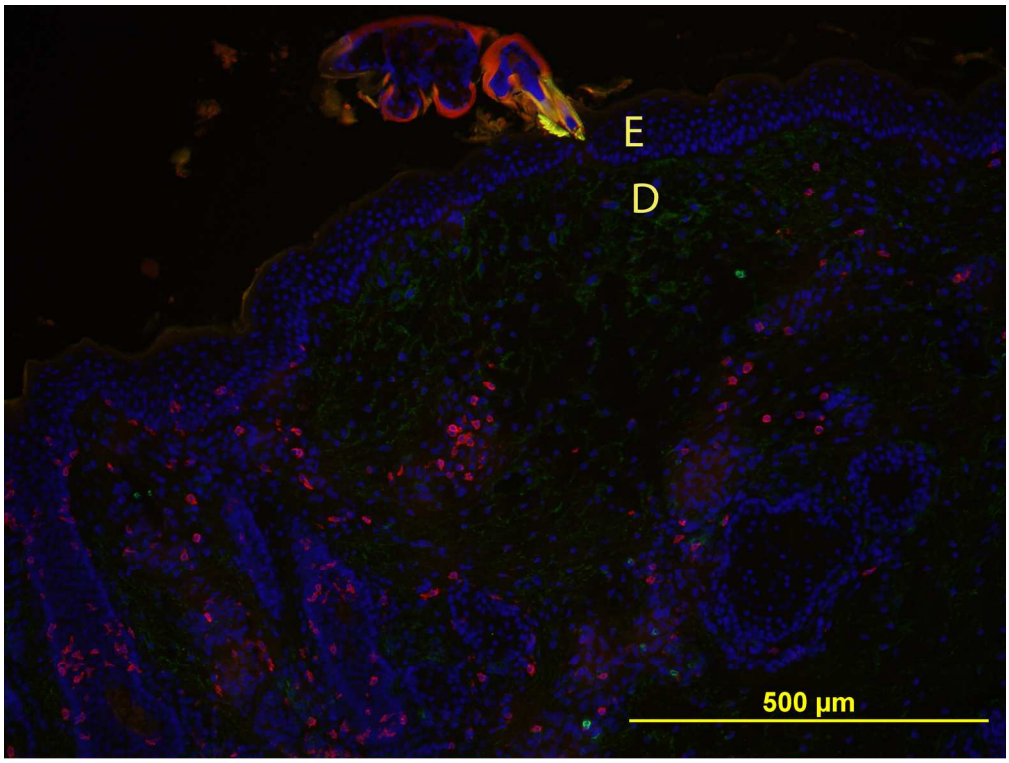

Fig. 2b

180x135mm (300 x 300 DPI)

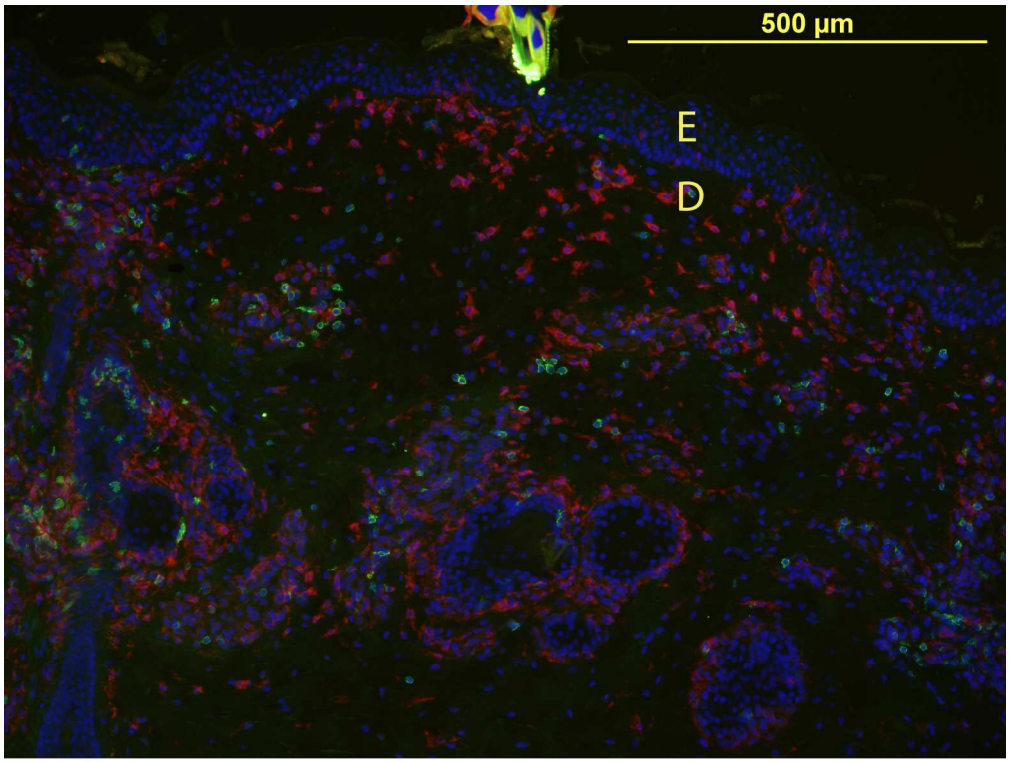

Fig. 2c

180x135mm (300 x 300 DPI)

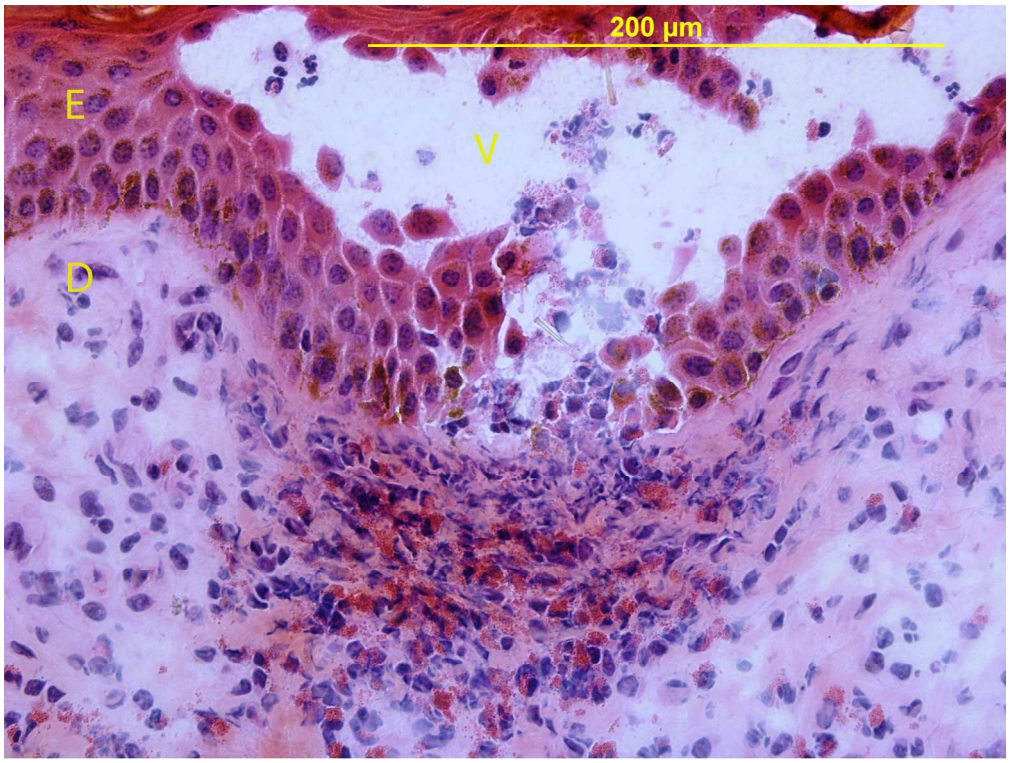

Fig. 3a

180x135mm (300 x 300 DPI)

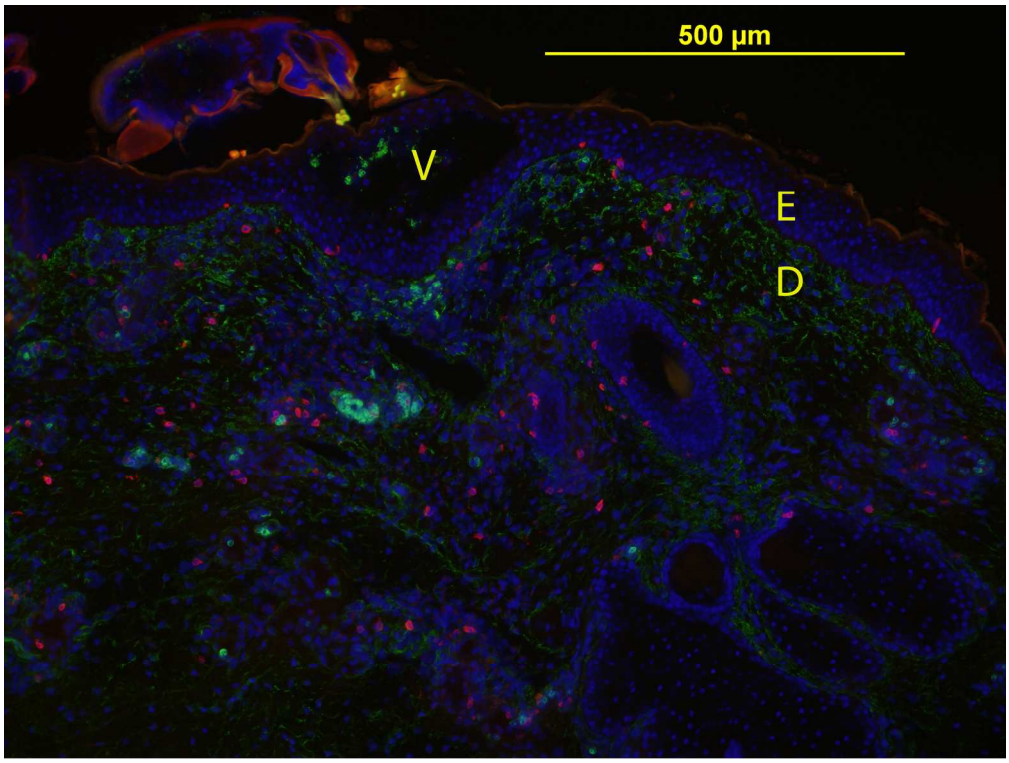

Fig. 3b

180x135mm (300 x 300 DPI)

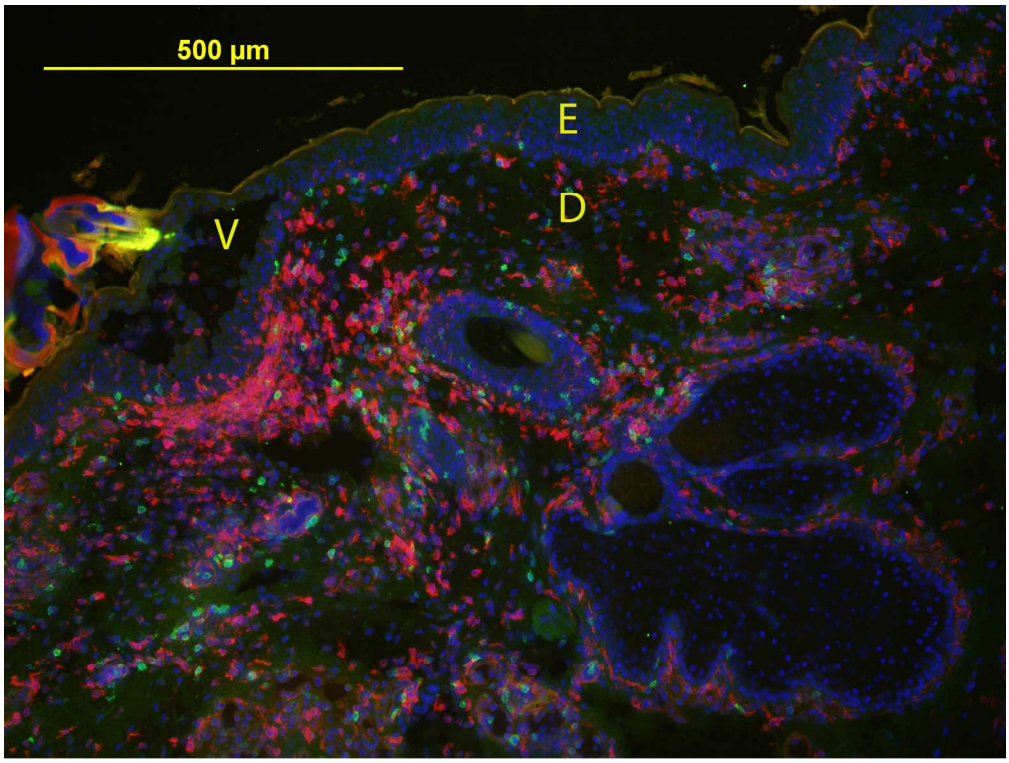

Fig. 3c

180x135mm (300 x 300 DPI)

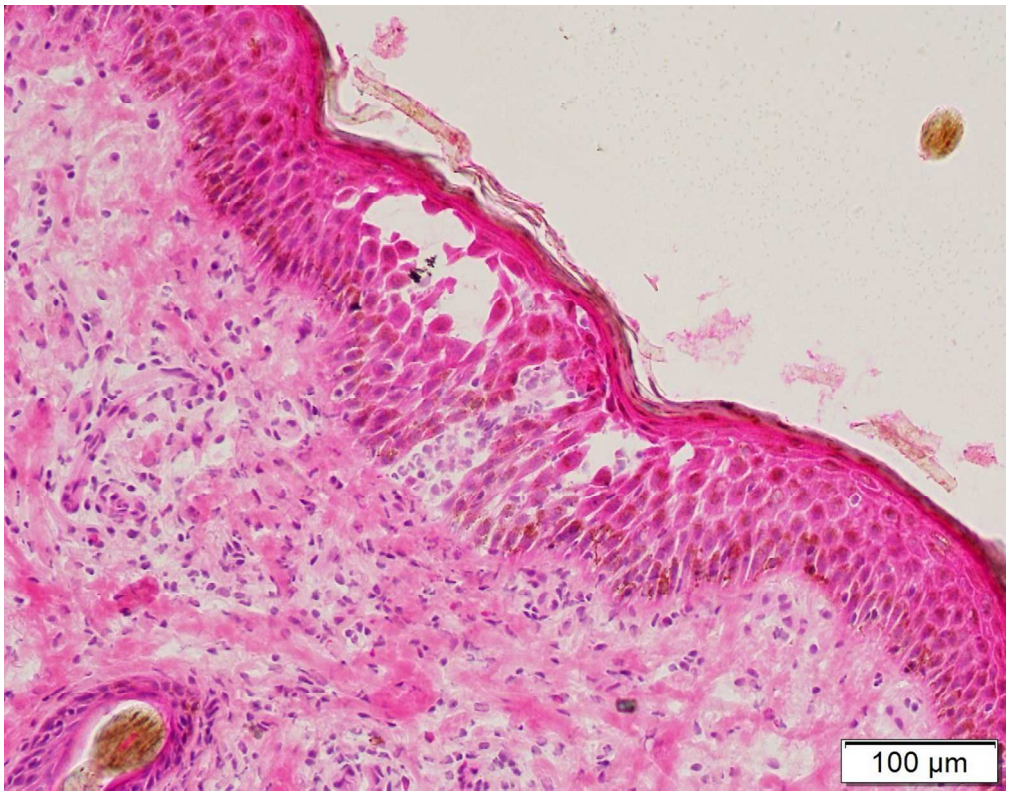

Fig. 4a

431x338mm (72 x 72 DPI)

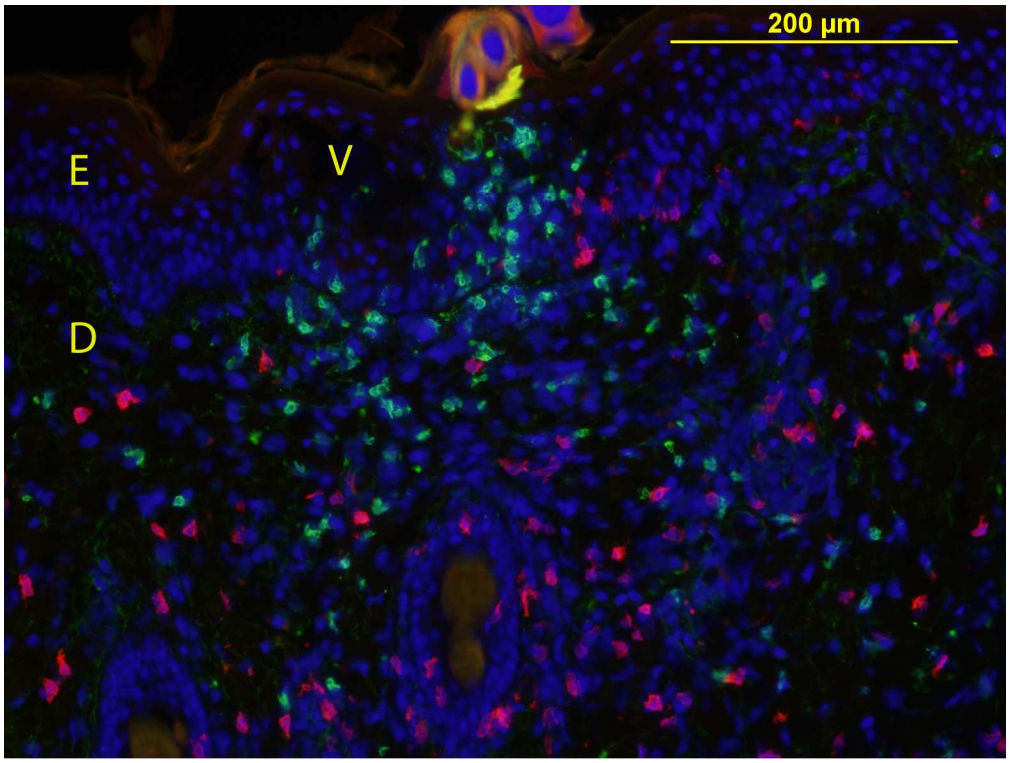

Fig. 4b

180x135mm (300 x 300 DPI)

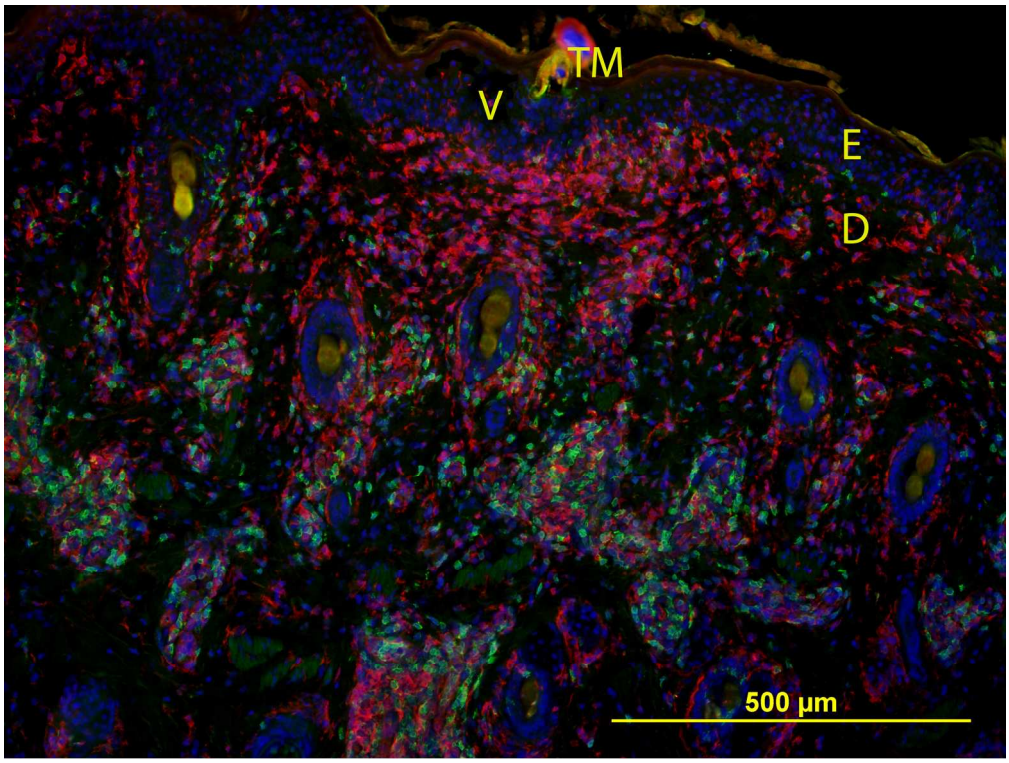

Fig. 4c

180x135mm (300 x 300 DPI)

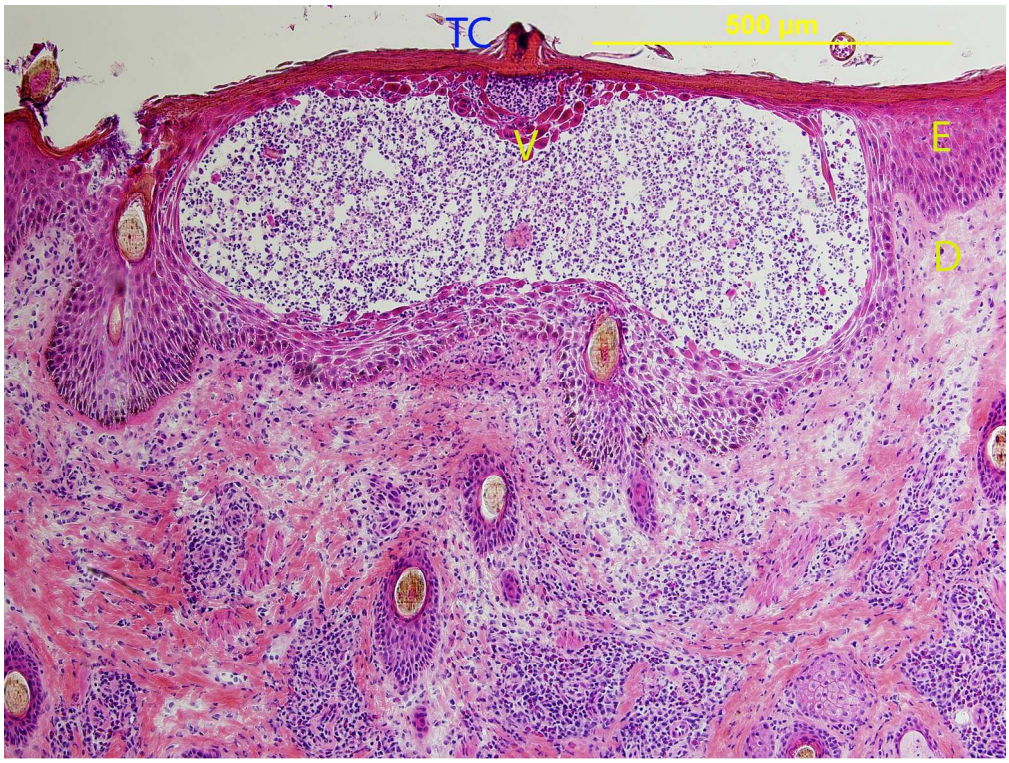

Fig. 5a

180x135mm (300 x 300 DPI)

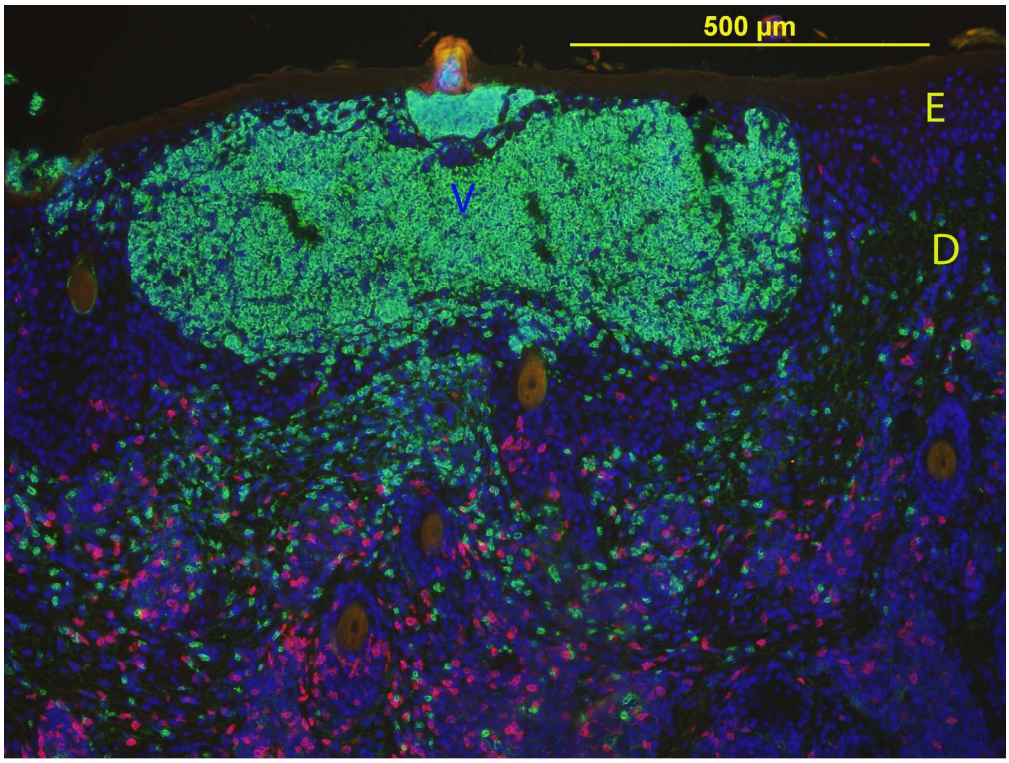

Fig. 5b

180x135mm (300 x 300 DPI)

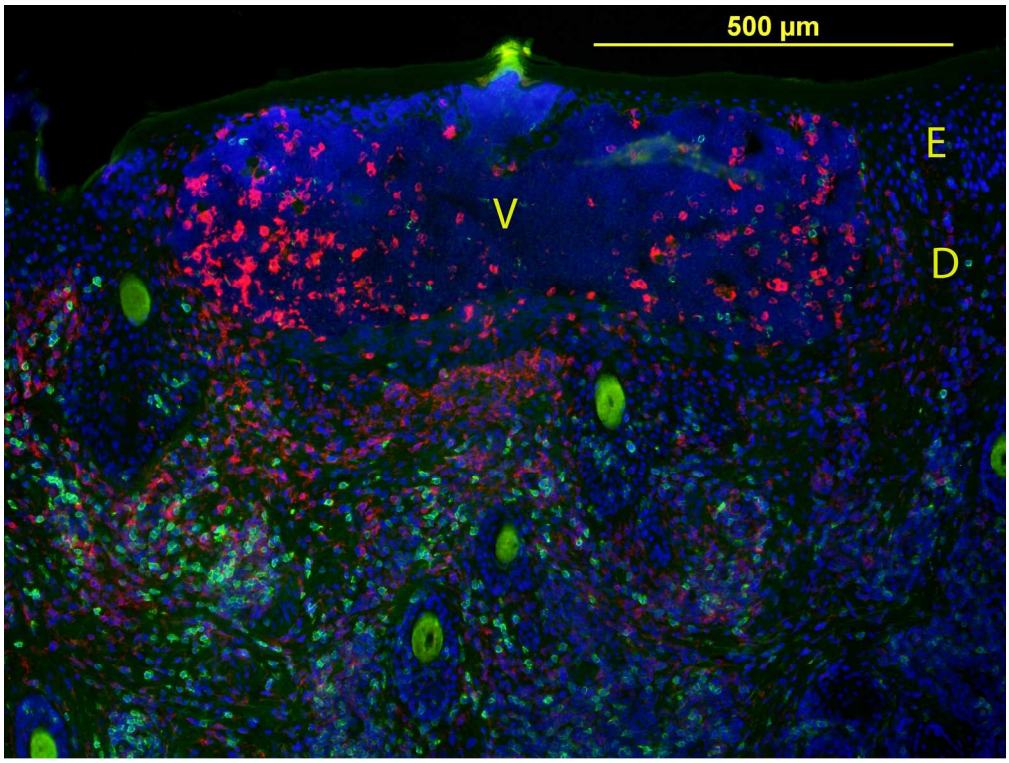

Fig. 5c

180x135mm (300 x 300 DPI)

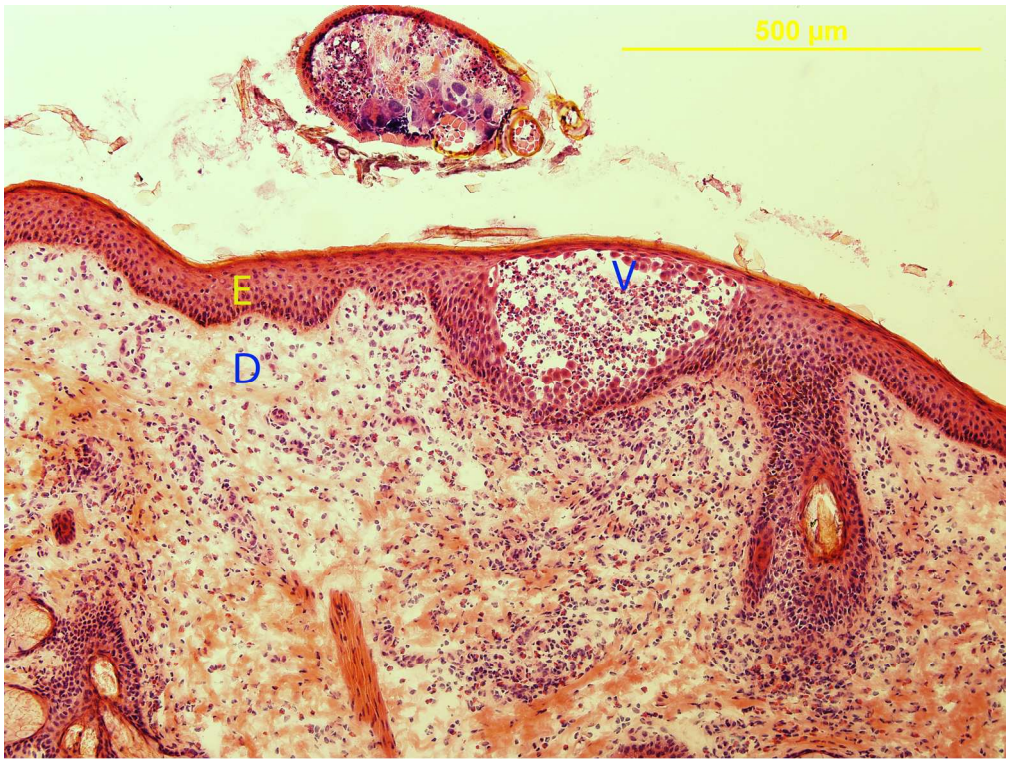

Fig. 6a

180x135mm (300 x 300 DPI)

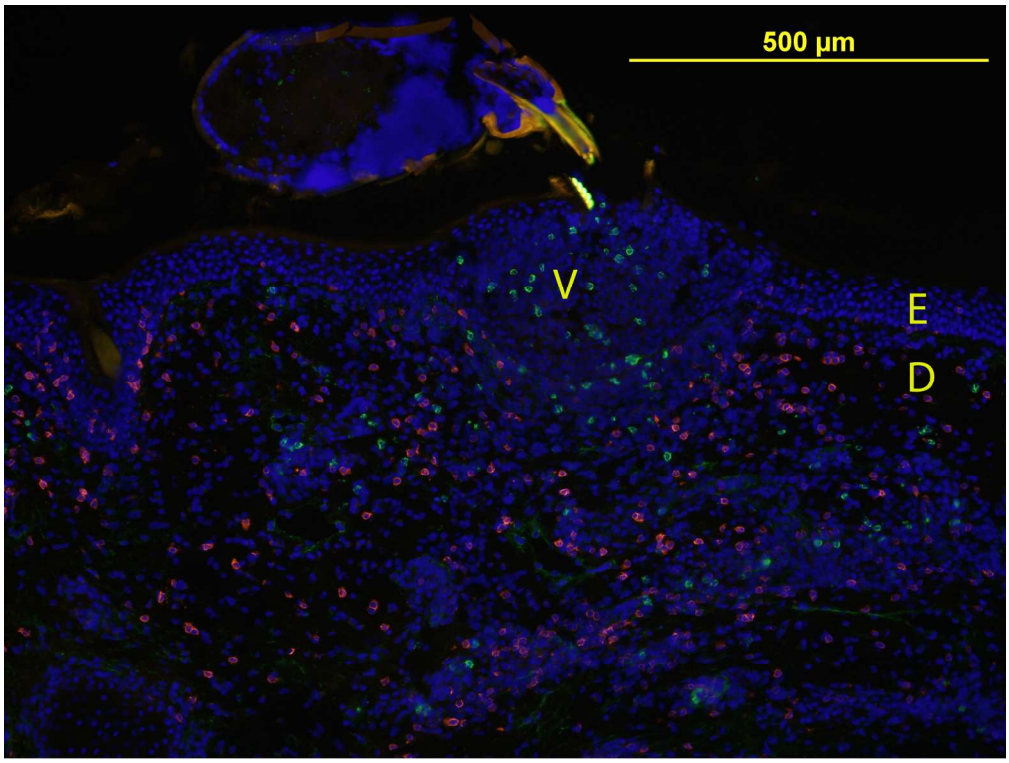

Fig. 6b

180x135mm (300 x 300 DPI)

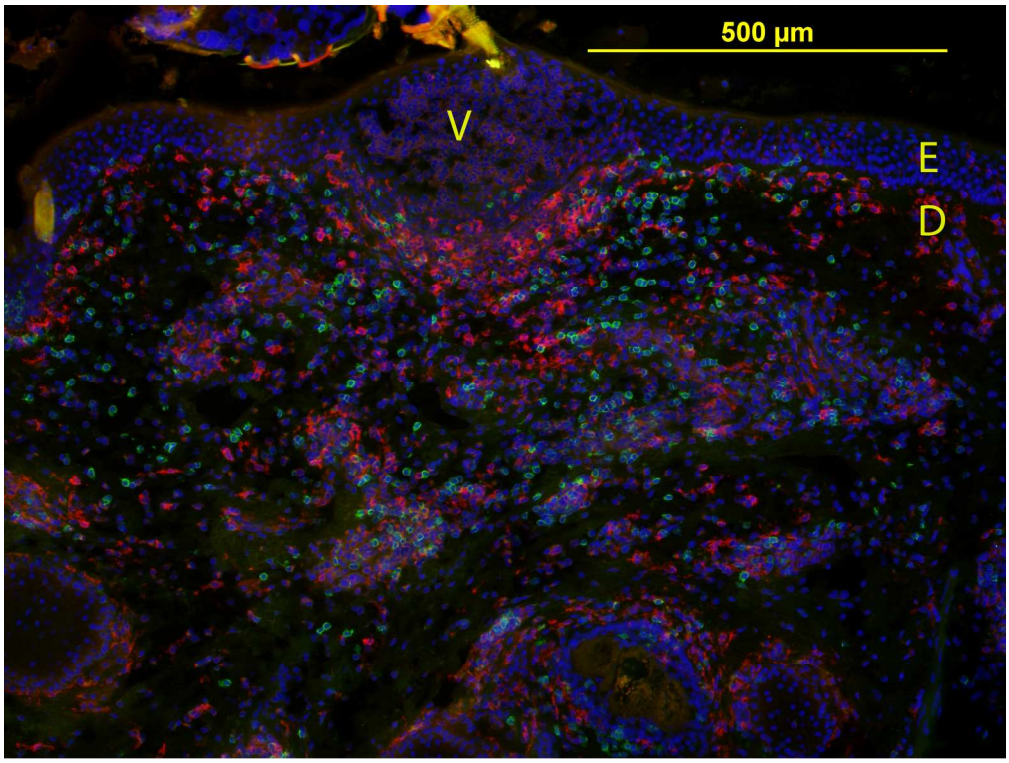

Fig. 6c

180x135mm (300 x 300 DPI)

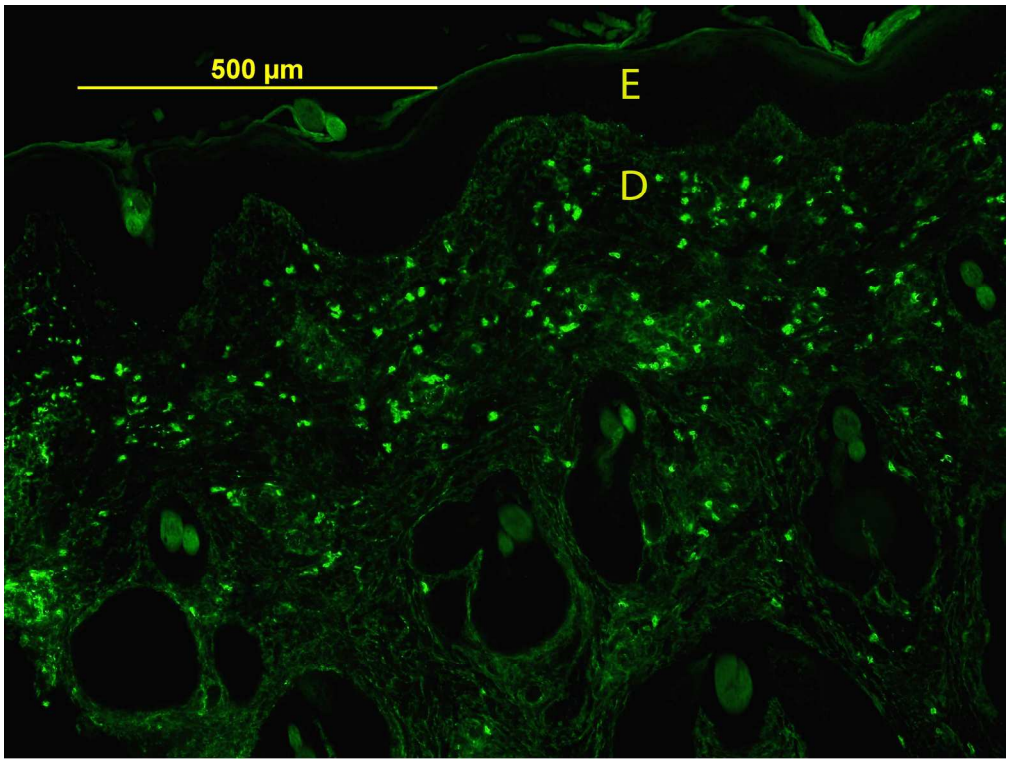

Fig. 7

180x135mm (300 x 300 DPI)

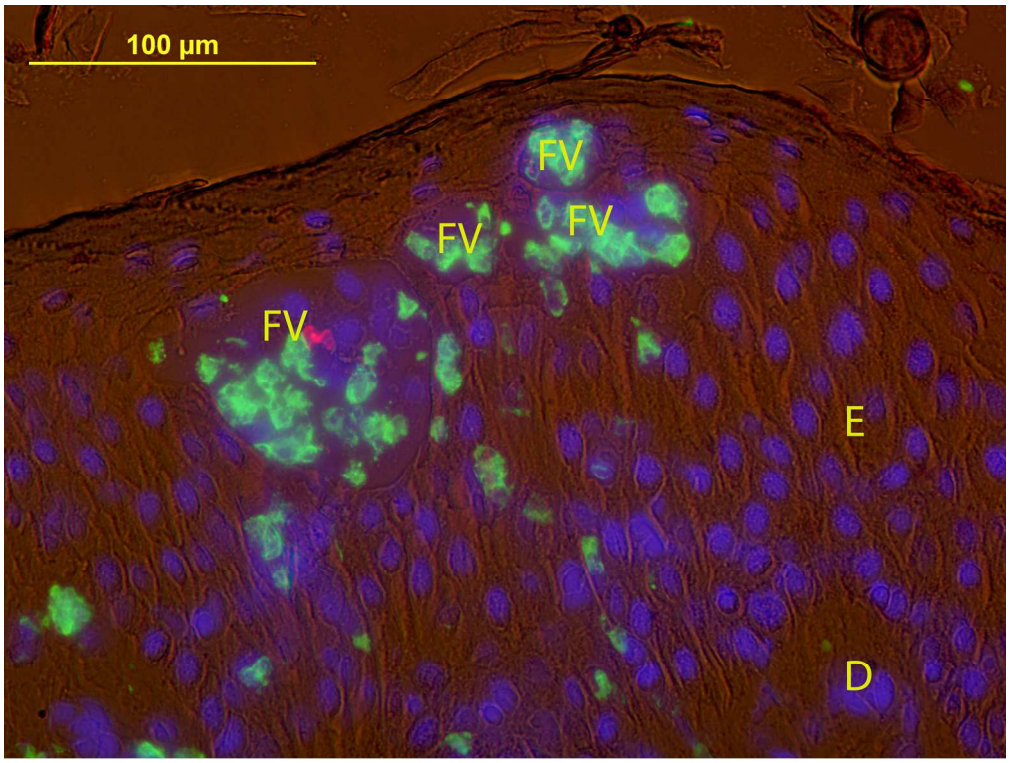

Fig. 8

180x135mm (300 x 300 DPI)

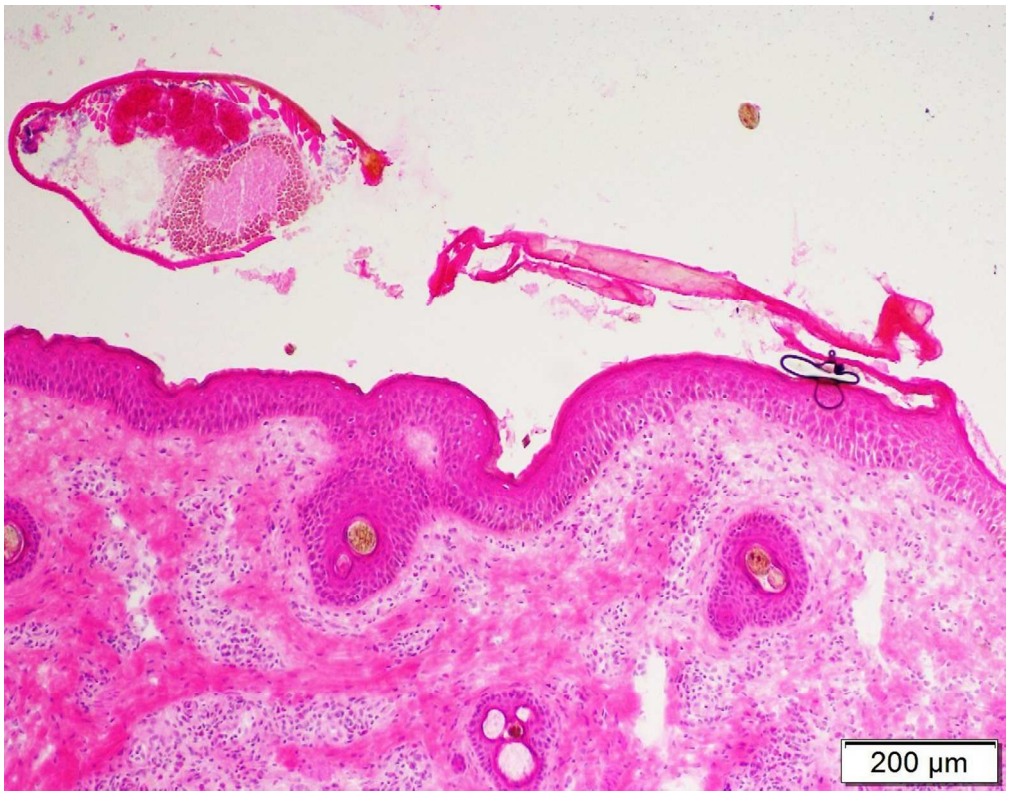

Fig. 9a

431x338mm (72 x 72 DPI)

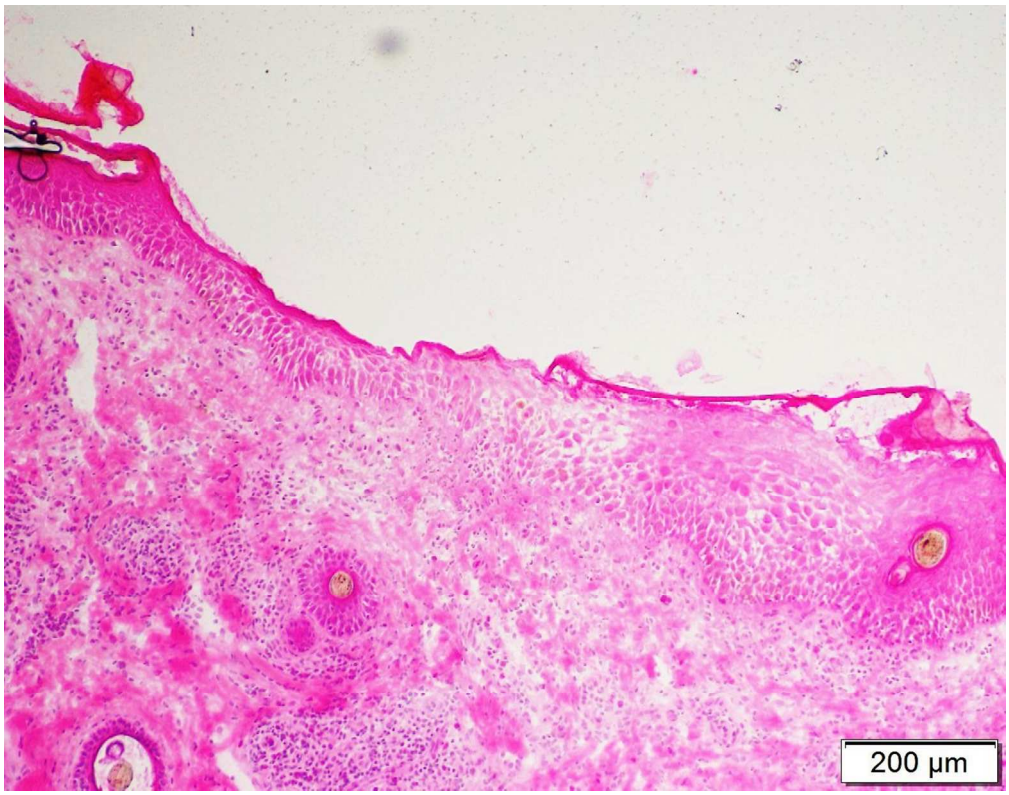

Fig. 9b

431x338mm (72 x 72 DPI)

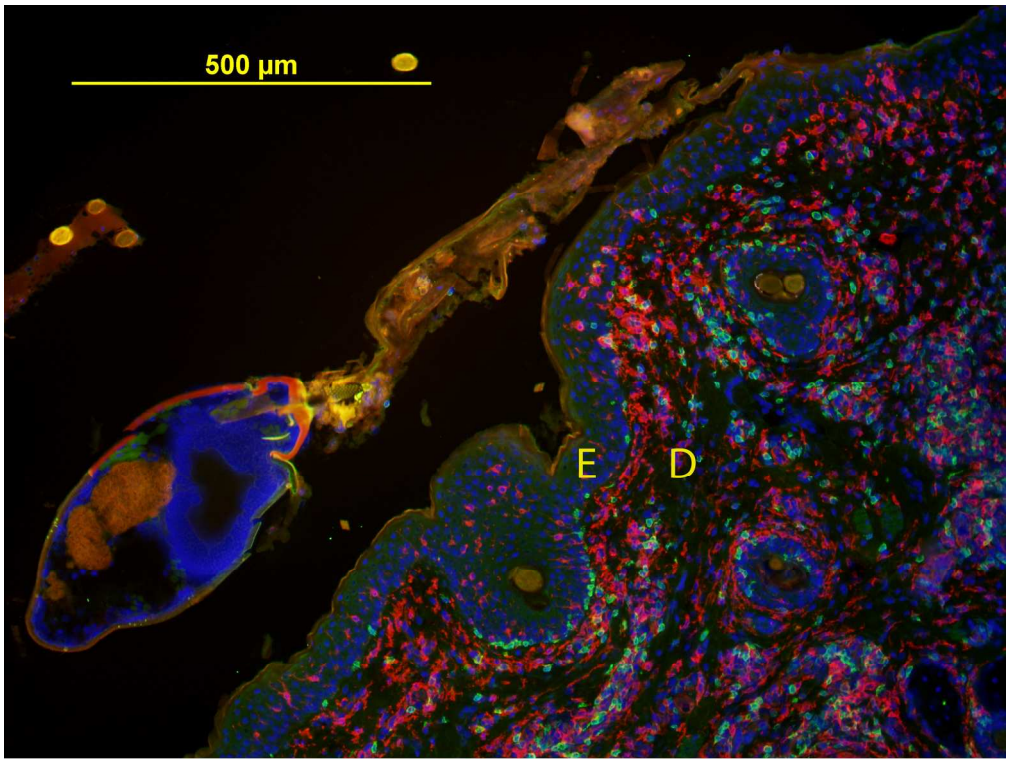

Fig. 9c

180x135mm (300 x 300 DPI)

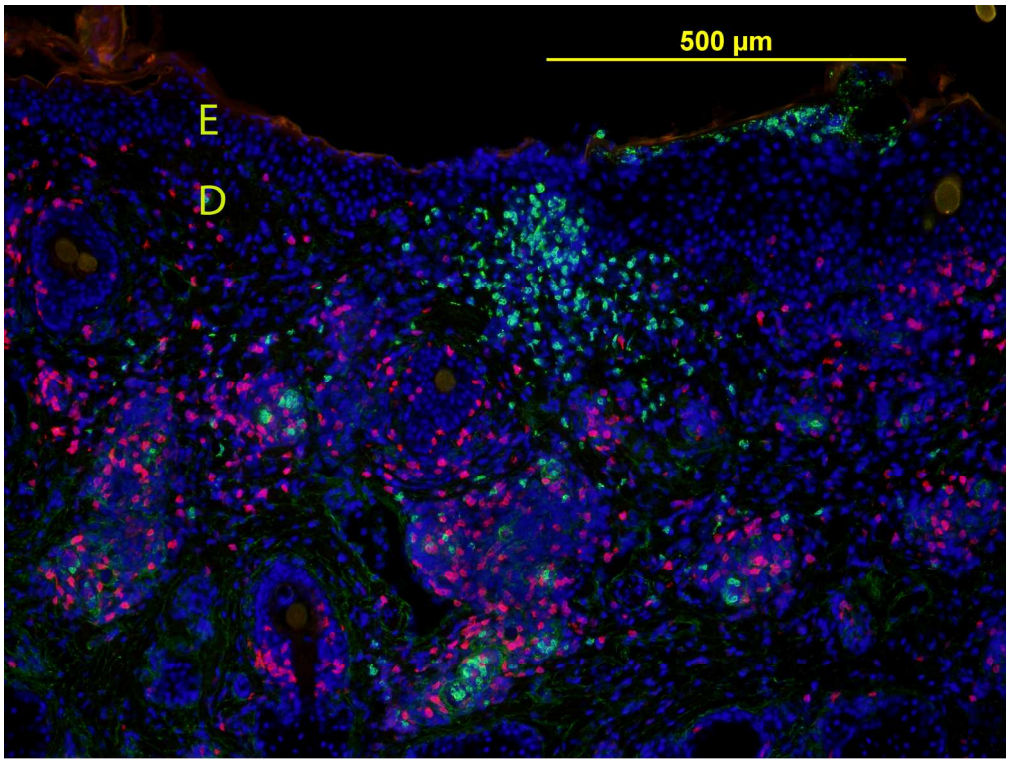

Fig. 9d

180x135mm (300 x 300 DPI)

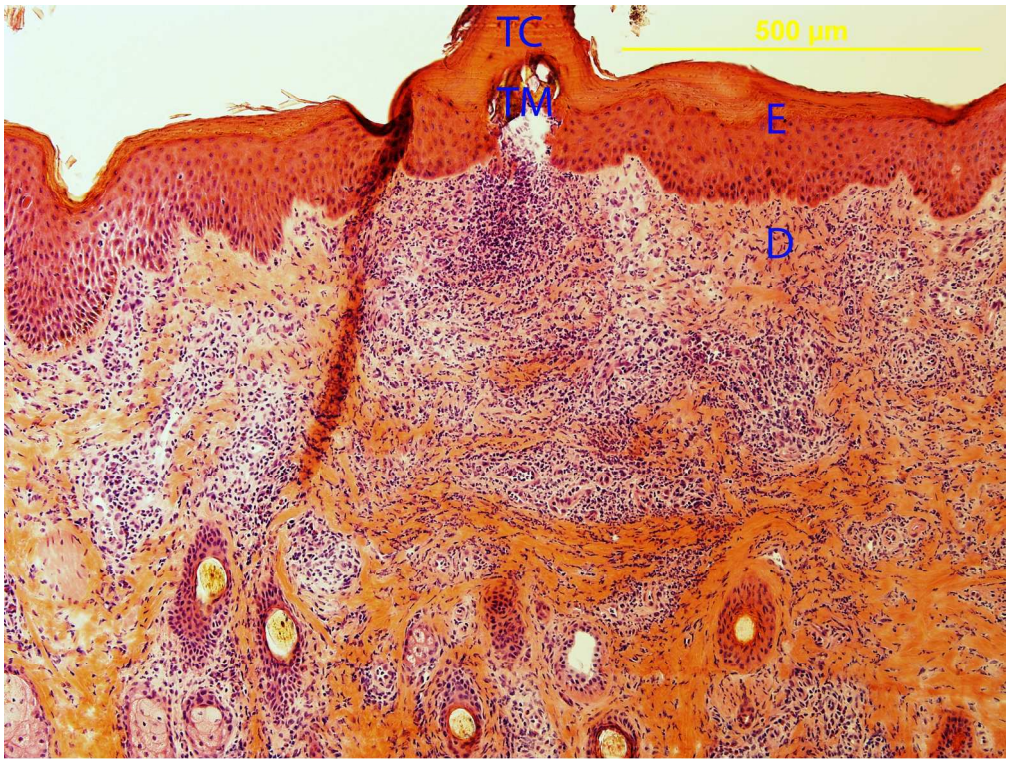

Fig. 10a

180x135mm (300 x 300 DPI)

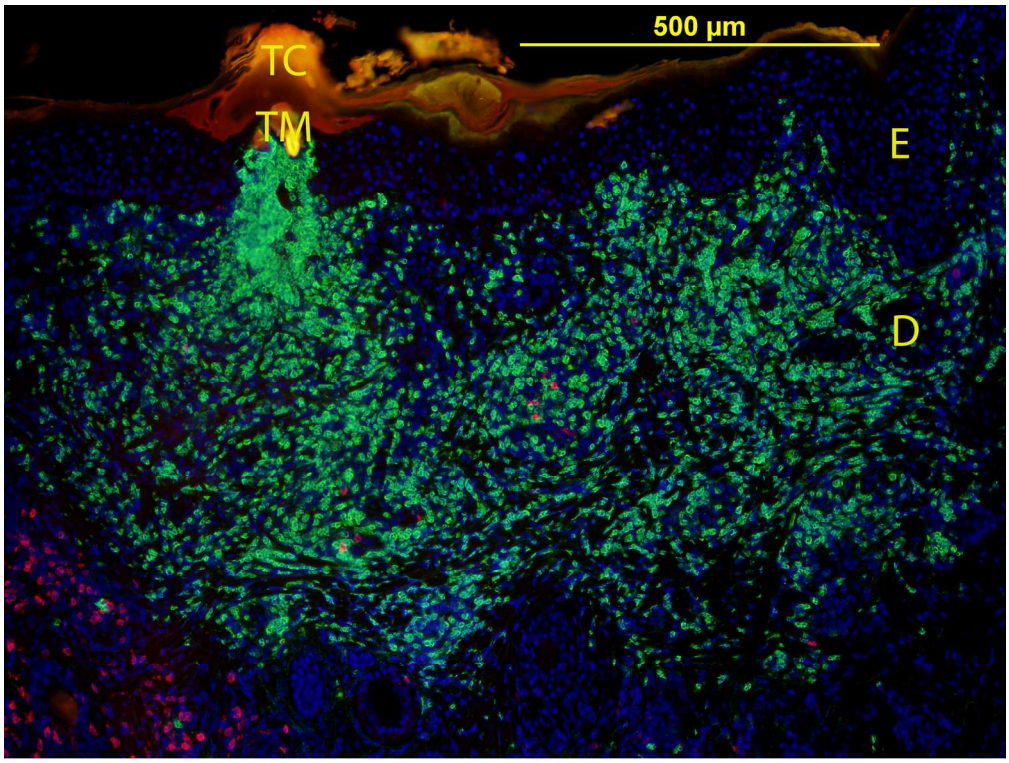

Fig. 10b

180x135mm (300 x 300 DPI)

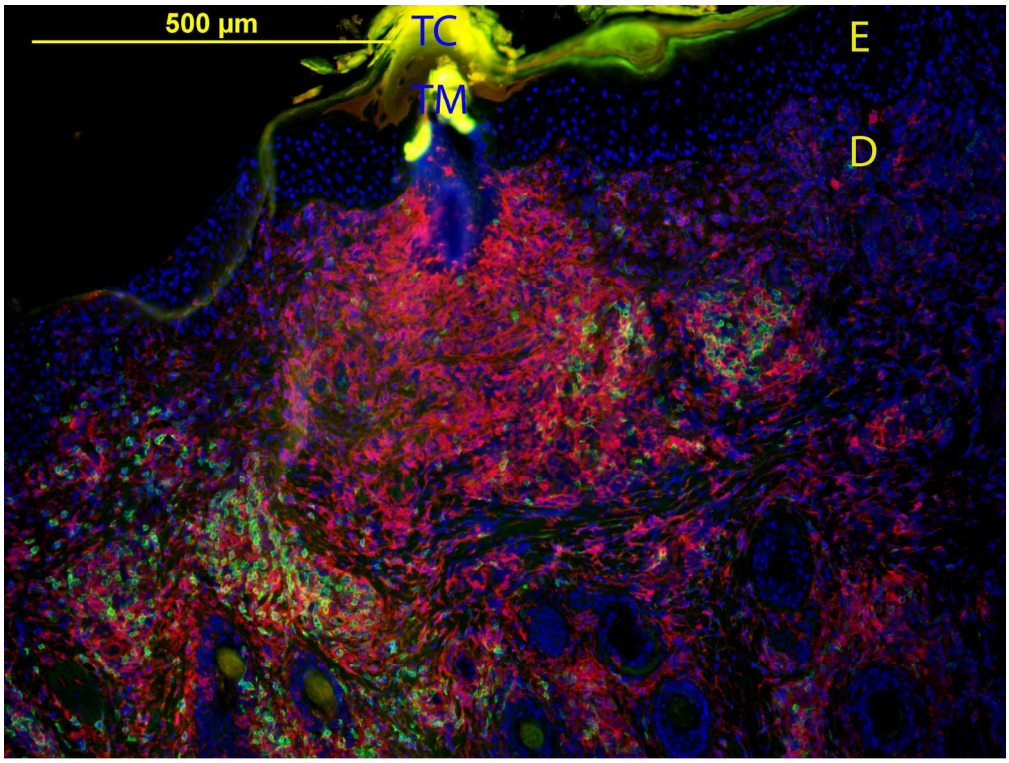

Fig. 10c

180x135mm (300 x 300 DPI)

Table 1 Monoclonal antibodies used to characterize cells infiltrating the skin areas around tick mouthparts

Monoclonal antibody designation	Source	Antigen specificity	Isotype	Cellular expression	Dilution used	Reference
CACTB51A	VMRD	CD45	IgG2a	Leukocytes	1/800	(45, 46)
Il-A116	VMRD	CD45RO	IgG3	Activated cells	1/400	(47)
MM1A	VMRD	CD3	IgG1	T cells	1/800	(48)
CH138	VMRD	Neutrophils	IgM	Neutrophils	1/400	(29, 46, 49)
MCA837G	AbD Serotec	CD8	IgG2a	T cytotoxic cells	1/50	(50, 51)
HM57	DakoCytomation	CD79ά	IgG1	B cells	1/100	(52)
IL-A29[a]	ILRI[b]	γδ form of the T cell receptor	IgG1	γδ T cells	1/25	(53)
IL-A21[a]	ILRI[b]	MHC class	IgG2a	Macrophages	1/200	(54)

		II antigen		, dendritic cells, B cells, activated T cells		
IL-A12[a]	ILRI[b]	CD4	IgG2a	T helper cells	1/25	(55)
IL-A111[a]	ILRI[b]	CD25	IgG1	Activated cells (IL2-R bearing cells)	1/25	(56)

[a] Monoclonal antibodies from tissue culture supernatant

[b] International Livestock Research Institute, Nairobi, Kenya

Table 2 The reactivity of the antibodies specific for CD45 and CD45RO epitopes with the skin leukocytes of cattle with different levels of tick resistance

Cow tag	**CD45**	**CD45RO**
B907-S	+	+
B797-S	+	+
B639-S	+	+
B629-S	-	+
B615-S	-	+
B607-S	-	-
B809-R	+	+
B825-R	-	-
B821-R	-	-
B783-R	-	-
B679-R	-	-
B501-R	-	+/-

Table 3 Type of reaction at tick attachment site in susceptible and resistant cattle

Type of reaction at tick attachment site	Susceptible cattle (6 tick attachments)	Resistant cattle (5 tick attachments)
Absence of any cell infiltration/reaction	1	1
Cellular infiltrations	1 (eosinophils, neutrophils & MHC class II antigen cells)	0
Empty intra-epidermal vesicle with no visible/obvious infiltrations around vesicle	0	1
Empty intra-epidermal vesicle with cellular infiltrations around vesicle	2 (neutrophils & MHC class II antigen cells adjacent to the vesicle)	2 (eosinophils & MHC class II antigen cells adjacent to the vesicle)
Intra-epidermal vesicles filled with cells	1 (neutrophils within vesicle and neutrophils and MHC class II antigen cells adjacent to the vesicle) 1 (neutrophils and eosinophils within vesicle)	1 (eosinophils within vesicles and eosinophils and MHC class II antigen cells adjacent to the vesicle)

Table 4 Inflammatory reaction in the skin of the cattle: parameters assessed in the epidermis and dermis and their scores

Type	Naive					Susceptible					Resistant				
Cow tag	B407	B605	B573	B507	B857	B629	B639	B797	B615	B607	B821	B679	B783	B825	B501
Epidermis															
Acanthosis	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Apoptosis/necrosis	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
Acantholysis	0	0	0	0	0	0	0	0	2	2	0	1	3	2	2
Micro-abscess	2	0	0	0	0	2	2	2	2	1	0	2	3	2	2
Subepidermal clefting	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Transepithelial leukocyte migration	2	0	0	0	0	2	3	1	2	2	0	2	3	2	2
Hyperkeratosis - ortho	0	0	0	0	0	2	2	1	1	1	0	1	0	1	1
Hyperkeratosis - para	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermis															
Oedema	0	0	0	0	0	1	2	1	1	2	1	2	2	2	2
Collagen degeneration	0	0	0	0	0	2	2	1	1	1	1	2	2	2	2
Vascular rexn/vasculitis	0	0	0	0	0	1	1	1	1	2	1	1	2	1	1
Transendothelial leukocyte migration	0	0	0	0	0	3	2	1	1	2	1	1	2	1	1
PMN/Eosinophil infiltrate	1	0	0	1	0	3	2	2	2	2	1	2	3	2	2

Mononuclear cell infiltrate	0	0	0	0	0	3	2	2	1	2	1	1	3	1	1
Mast cell infiltrate	0	0	0	0	0	2	2	1	1	2	1	1	2	1	1
Total	**5**	**0**	**0**	**1**	**0**	**23**	**22**	**13**	**16**	**19**	**7**	**16**	**25**	**17**	**17**