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CD markers of camel (*Camelus dromedarius*) intestine naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*: Distinct expression of Madcam-1 and CX3CR1

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Abstract: *Mycobacteriumavium* subsp. *paratuberculosis* (MAP) infection in camel requires extensive research, particularly the immune responses in the intestine. This study aimed to investigate the nature of the cellular populations that are driven by the immunopathological responses in the camel intestine infected with MAP at different ages. Immunohistochemical staining was carried out on tissues obtained from naturally infected young (5–10 years old) and older (12–15 years old) camels. The staining of the tissues, ileum, mesenteric lymph node, jejunum, and supramammary lymph nodes, with anti-CD3$^+$, CD4$^+$, CD8$^+$, CD25$^+$, CD11c$^+$, CD14$^+$, WC1$^+$, CX3CR1, and Madcam-1 monoclonal antibodies revealed high expression of the molecules CD8$^+$, CD25$^+$, CD11c$^+$, CD14$^+$, WC1$^+$, CX3CR1, and Madcam-1 in the ileum and mesenteric lymph node of the infected older camels. The results indicated the recruitment of CD8$^+$ lymphocytes, CD14$^+$ macrophages, and CD11c$^+$ dendritic cells to the ileal lamina propria. High expression of CX3CR1 could indicate a vital role for this special macrophage phenotype in the ileal lamina propria in maintaining intestinal homeostasis. Madcam-1 expression could have an essential role in defining the nature of the recruited cells to the site of the infection. Expression of CX3CR1 and Madcam-1 is a novel finding that merits further attention and pursuit to reveal their significance in the immune responses to MAP in the camel's intestine.

Key words: Johne's disease, paratuberculosis, mycobacterium, camel, ileum, mesenteric lymph node

1. Introduction

Mycobacterium avium ssp. *paratuberculosis* (MAP) causes Johne's disease in domestic and wild ruminants such as cattle, sheep, goats, deer, antelope, and bison worldwide [1]. In Saudi Arabia, Johne's disease has been reported in sheep, goat, dairy cattle, and camel [2–5]. MAP infection has also been reported in wild nonruminant animals such as rabbit, foxes, stoats, and weasels [6].

The host's adaptive immune responses to MAP infection is somewhat paradoxical [7]. Despite extensive research on the pathogenesis of MAP infection, the detailed mechanisms by which MAP maintains its persistence and mediates the immunosuppressive status of the host are still unknown. However, it has been speculated that MAP initiates strategies at the early stage of the infection to maintain its persistence in the host mainly based on manipulation of the host's lipid metabolism, leading to the accumulation of cholesterol in the infected host cells [8,9].

Several suggestions have been proposed on the major tissues from which MAP reaches the intestine [10–12]. Initially, tonsils were proposed as the main site from which MAP get access to the host's blood circulation [12]. However, experimental infection studies have proven that the intestine is the primary MAP site of entry, with possible involvement of the mesenteric lymph node (MLN) [11,12]. The data have indicated that MAP is capable of suppressing immune responses in the intestine, interfering with mononuclear and polymorphonuclear cell migration as early as 12 h postinfection [13]. The site of the infection is characterized by the accumulation of macrophages and dendritic cells (DCs). The spread of infection has also been recorded through the migration of the infected macrophages and DCs to the intestinal lumen [13].

The presence of MAP in the intestine stimulates a wide range of cellular populations that express different CD markers [14–17]. This work has addressed the markers that

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are expressed by the cellular population that play a pivotal role in the immune and inflammatory responses. The markers are $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, $CD11c^+$, $CD14^+$, workshop cluster-1^+ ($WC1^+$), CX3CR1, and mucosal vascular addressin cell adhesion molecule-1 (Madcam-1).

$CD3^+$ is the moiety of the T-cell receptor (TCR) complex; it is essential for TCR expression and signal transduction [18].

$CD4^+$ is a transmembrane glycoprotein member of the immunoglobulin (Ig) superfamily. $CD4^+$ acts as an MHC-II antigen coreceptor which plays a role in stabilizing of MHC/TCR complex. $CD4^+$ is mainly expressed in T-helper cells, T thymocytes, monocytes, granulocytes, and macrophages [18].

$CD8^+$ is a member of the Ig superfamily which acts as a coreceptor for the MHC-I antigen that stabilizes the MHC-I/peptide complex. $CD8^+$ is mainly expressed in T-cytotoxic cells, γδ cells, and natural killer (NK) cells [18].

$CD25^+$ is an integral membrane glycoprotein which acts as a low affinity interleukine-2 (IL-2) receptor. $CD4^+$ cells which coexpress $CD25^+$ are known as regulatory cells that limit inflammatory responses. B cells, monocytes, and macrophages also express $CD25^+$ [18].

$CD11c^+$ is an integral membrane glycoprotein which acts as integrin in combination with $CD18^+$ ($CD11c^+$/$CD18^+$). $CD11c^+$/$CD18^+$ is a type 4 complement receptor which acts as a chemotactic and adhesion factor for monocytes. $CD11c^+$ is expressed in monocytes, macrophages, and NK cells [18].

$CD14^+$ is a phosphatidylinositol glycan-linked membrane protein which mainly acts as a high-affinity lipopolysaccharide receptor [19].

$WC1^+$ is a member of the transmembrane glycoprotein of the scavenger receptor cysteine rich family (SRCR), a family that is usually expressed on the γδ T cells. The $WC1^+$ coreceptor is essential in mediating the activation of γδ cells [20,21].

Madcam-1 is a ligand for the integrin α4β7 Peyer's patches adhesion molecule-1 (LPMA-1) and the L-selectin (CD62L). Madcam-1 is a crucial molecule in the cells trafficking to the intestine and mesenteric lymph node (MLN), which defines the nature of the cellular population involved in the innate and adaptive immunity of human and mouse intestine [22,23]. The expression of Madcam-1 and its ligand LPMA-1 in camel tissues has been recorded [24–26].

The chemokine receptor CX3CR1 is expressed by a special lineage of intestinal macrophages [27]. Despite wide debate on the nature of the cells expressing this receptor, recent findings have shown categorically that they are a lineage of macrophages that reside in the intestinal lamina propria (LP) [27].

There is a lack of evidence elaborating on the pathogenesis of MAP infection in camel at different stages. The majority of the studies that have described the clinical and pathological changes of MAP infection have been carried out on camels at the early or advanced clinical stage of the disease [28–33]. Hence, explicit information about MAP infection in camel requires intensive research, particularly on the intestine as the major site of MAP persistence. Therefore, this study has embarked on exploring the types and subtypes of the cells recruited to the camel intestine and the nature of the CD markers expressed during the MAP infection.

2. Materials and methods

2.1. Animals

One hundred and seventy-six (176) female camels were classified into 2 groups according to their age. The younger group consisted of 5–10-year-old camels (58 animals), and the second group consisted of 12–15-year-old camels (118 animals). All animals were slaughtered at Al-Omran Slaughterhouse following the Ethical Guide for Handling Animals in the Slaughterhouses (Ministry Municipal and Rural Affairs, Saudi Arabia)

2.1.1. Identifying the infected animals

Based on previously described clinical signs [32], the suspected animals were isolated, and the following tests were carried out to confirm infection.

ELISA: Blood samples were collected from all 176 animals. The serum samples were tested for the presence of the anti-MAP antibodies using commercial ELISA (LSIVet Ruminant Serum Paratuberculosis Screening ELISA Kit, France). The samples were examined following the manufacturer's directions. The ELISA plates were read using a BioTek ELX800 Absorbance Reader (BioTek Instruments Inc., Winooski, VT, USA) [4].

Ziehl-Neelsen staining: Ziehl-Neelsen staining was performed on fecal samples taken from the recta of animals from which the tissue samples were collected [34].

Histopathological changes: The postmortem profile Rectum was based on the thickness and redness of the MLN, ileum, and jejunum [32].

2.2. Tissue samples

Tissue specimens were collected from the supramammary lymph node (SLN), MLN, ileum, and jejunum of all 176 animals. Samples were immediately fixed in 4% paraformaldehyde in PBS (pH 7.3). After 48 h fixation, tissues were embedded in paraplast Tissue Embedding Media (Leica Microsystems, St. Louis, MO, USA) and processed using an automated tissue processor (TP 1020, Leica, Wetzlar, Germany). Tissue was then sectioned at 5-μm thickness with a rotary microtome (RM 2135, Leica). The sections were floated in a warm water bath

(41°C) and then mounted onto Superfrost Plus glass slides (Thermo Fisher Scientific, Waltham, MA, USA) for routine histological and immunohistochemical techniques.

2.2.1. Conventional histological staining

Standard hematoxylin and eosin (H&E) protocol according to Bancroft and Cook]34[was used to investigate general histological structures and histopathological examinations of the tissue samples.

2.2.2. Immunohistochemistry

The expressions of the markers $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, $CD11c^+$, $CD14^+$, $WC1^+$, CX3CR1, and Madcam-1 were evaluated on the paraformaldehyde-fixed, paraffin-embedded ileum, jejunum, MLN, and SLN tissues. Immunohistochemical staining were performed on 5-µm cross-sections using a goat anti-mouse HRP/DAB Detection IHC Kit (Abcam Plc, Cambridge, UK). The staining was performed according to the manufacturer's directions. Target proteins were detected with primary antibodies (types, sources, host, reactivity, and clonality) as shown in Table 1. Briefly, sections were dewaxed, rehydrated, and incubated overnight with primary antibodies at 4°C, while the rest of the incubations were performed at room temperature. After incubation with the primary antibodies, sections were then washed several times followed by incubation with biotinylated goat anti-mouse for 10 min at room temperature. After washing with PBS, the sections were incubated with streptavidin peroxidase and incubated for 10 min at room temperature. After washing, visualization was achieved by immersing sections in freshly prepared DAB chromogen solution until the desired stain intensity developed. For negative controls, primary antibodies were substituted with PBS while the rest of the procedures were maintained.

Following previously described criteria for scoring [35], staining intensities were scored visually (no expression, low expression, moderate expression, and high expression) by 2 independent observers; a high expression is the highest score and no expression is comparable to the negative control.

3. Results

3.1. The detection of anti-MAP antibodies with ELISA

According to antemortem, postmortem, Ziehl-Neelsen staining, and ELISA tests, the analysis of the 176 serum samples revealed that 13 camels had positive reactions: 5 camels from the young group (5–10 years old) and 8 camels from the older group (12–15 years old).

3.2. The Immunohistochemical staining

The cellular distribution in ileum: The general H&E stain structural features of the ileum of normal and infected camels were posted for the exploration of any immunopositive cells within the ileum in the subsequent sections (Figure 1a).

$CD3^+$: The $CD3^+$ marker was highly expressed around the follicles located beneath the villi of ileum in the normal young camels. The $CD3^+$ marker expression, however, was low in the ileum of the infected older camels. Normal older and infected young camels had low or no expression of $CD3^+$ (Figure 1b).

$CD4^+$: The $CD4^+$ marker was highly expressed in the villi and submucosal region of the ileum of the normal older camels. However, no $CD4^+$ expression was noticed in the rest of the ileal samples of infected older and normal young camels (Figure 1c).

$CD8^+$: The cells with high expression of $CD8^+$ were only detected in the ileum of the infected older camels. The ileal tissue of the infected young camels and the normal camels indicated no expression of the $CD8^+$ (Figure 1d).

$CD25^+$: The cells expressing $CD25^+$ were highly present in the ileum of the normal young and older camels as well as of the infected older and younger camels (Figure 1e).

$WC1^+$: The ileum of the normal young camels and the ileal tissue of the infected older camels showed high levels of The γδ cells expressing $WC1^+$. However, the ileum of

Table 1. The markers for the detection of the camel CD markers in intestine.

Markers	Clone	Isotype	Source
Mouse anti-human $CD3^+$	UCHT1	IgG1	www.origene.com
Mouse anti-bovine $WC1^+$	CC101	IgG2a	www.origene.com
Mouse anti-bovine $CD4^+$	CC30	IgG1	www.origene.com
Mouse anti-human $CD25^+$	OX-39	IgG1	www.origene.com
Mouse anti-human Madcam-1	17F5	IgG1	www.genetex.com
Mouse anti-human CX3CR1	8E10.D9	IgG1	www.abcam.com/abpromise
Mouse anti bovine $CD8^+$	CC63	IgG2a	www.MyBioSource.com
Mouse anti-human $CD14^+$	OTI4H4	IgG1	www.origene.com
Mouse anti human $CD11c^+$	ITGAX	IgG2b, kappa	www.MyBioSource.com

the normal older camels showed moderate expression of WC1+, whereas no expression was seen in the ileum of the infected young camels (Figure 2a).

CD11c+: CD11c+ were highly expressed in the ileum of infected camels only (Figure 2b).

CD14 +: This marker was highly expressed only in the infected older camels. Its expression in the rest of the groups was negative (no expression) (Figure 2c).

CX3CR1: The chemokine marker showed high expression in the infected older camels, while its expression in the rest of the groups was negative (no expression) (Figure 2d).

Madcam-1: Madcam-1 was highly expressed in both infected groups while its expression was moderate in the normal older camels (Figure 2e).

The cellular distribution in MLN

H&E stain: The general structural features of the MLN of normal and infected camels were posted for the exploration of any immunopositive cells within the MLN in the subsequent sections (Figure 3a).

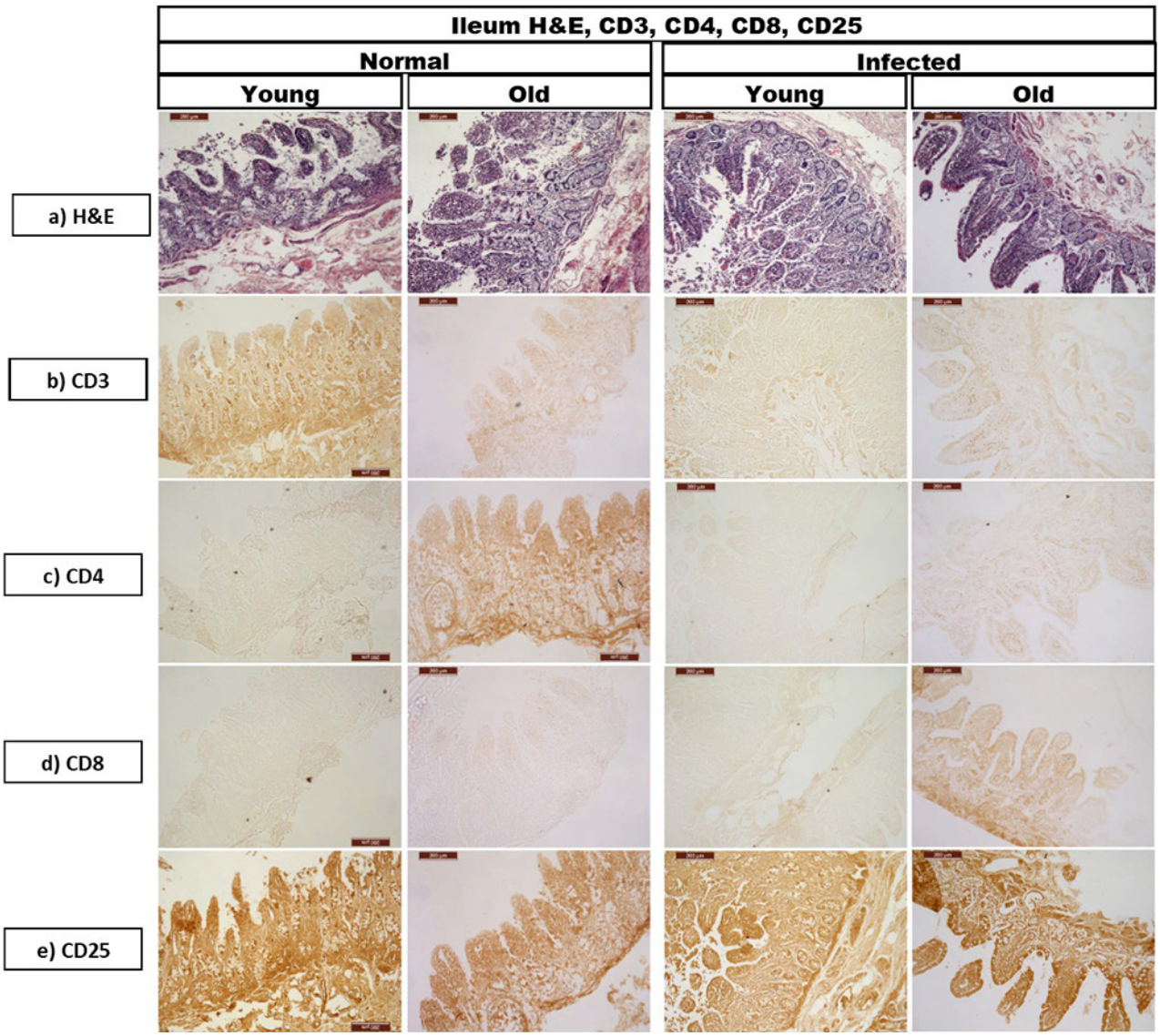

Figure 1. Histological sections from the ileum of the young (5–10 years-old) and old (12–15 years-old) camels stained with (a) H&E; (b-e) Immunohistochemical detection of CD3+, CD4+, CD8+& CD25+, respectively. Notice the extensive CD3 expression around the ileum of the young normal, minor in infected old, while no expression could be detected in old normal and young-infected camels (b). CD4+ marker was extensively expressed in the ileum of the old-normal camels, while no expression was noticed in the rest of the ileal samples (c). CD8+ was only detected in the ileum of the old-infected camels (d). CD25+ was extensively present in the ileum of all groups (e). (Scale bar: 200 µm).

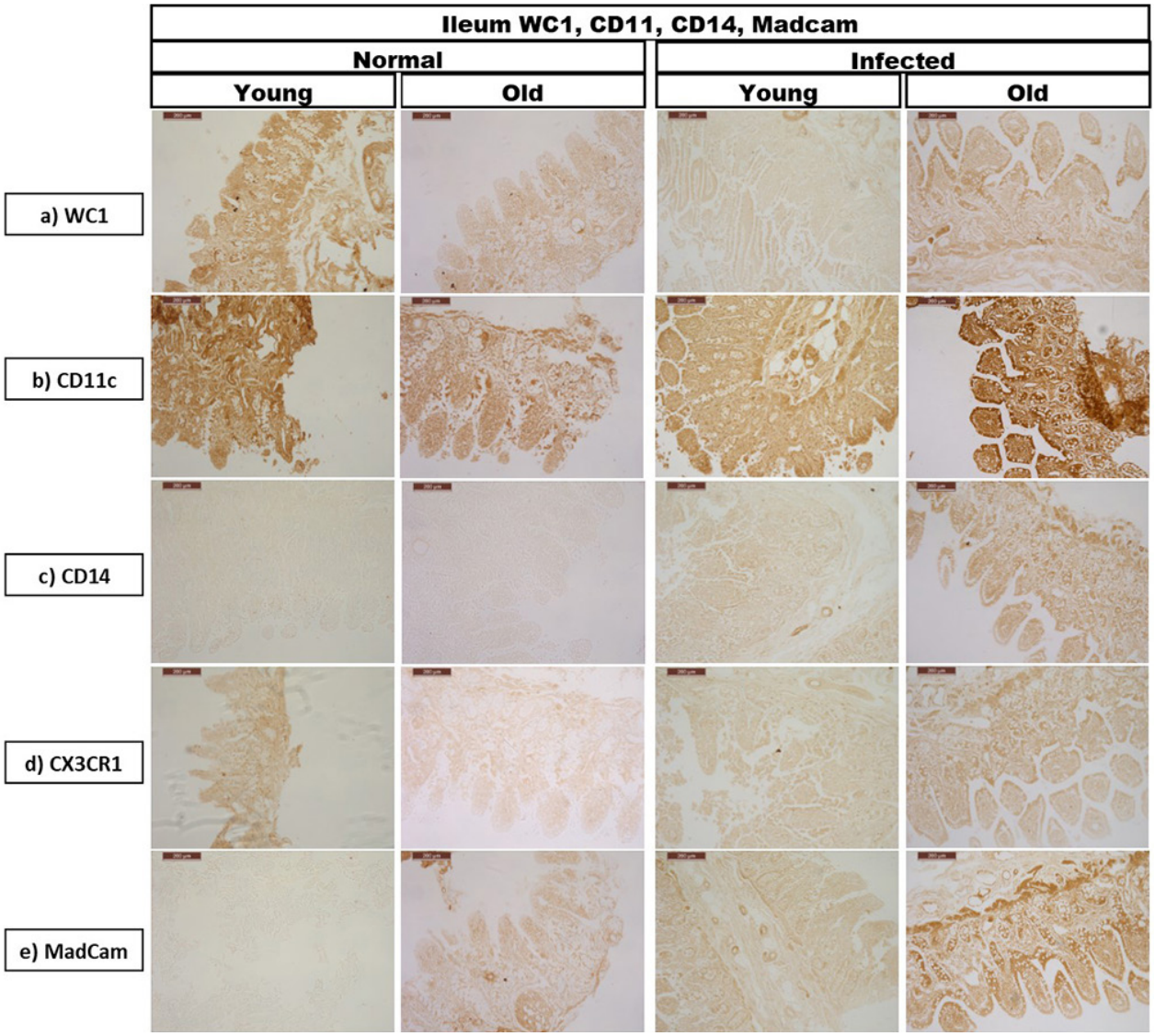

Figure 2. (a-e) Immunohistochemical detection of WC1$^+$, CX3CR1, CD11c$^+$, CD14$^+$ and Madcam-1 in ileum of different camel groups. Notice the extensive expression of WC1$^+$ in the ileum of the young-normal and old-infected camels, whereas slight expression in the old normal and no expression in the young infected camels (a). CD11c$^+$ was expressed overwhelmingly in the ileum of infected camels only (b). CD14 was distinctively expressed only in the old-infected camels (c). CX3CR1showed extensive expression in the old-infected camels, while its expression in the rest of the groups was minor (d). Madcam-1 was significantly expressed in the infected groups and its expression was obvious in the old normal camels (e). (Scale bar: 200μm).

CD3$^+$: It was highly expressed on the cells in the MLN of the normal young and infected older camels (Figure 3b).

CD4$^+$: No expression in any of the camel groups (Figure 3c).

CD8$^+$: The CD8$^+$ was highly expressed in the cells of all of the groups, while the MLN of the infected young camels indicated no expression (Figure 3d).

CD25$^+$: The expression of CD25$^+$ is similar to the expression of CD8$^+$ in MLN (Figure 3e).

WC1$^+$: The γδ cells expressing WC1$^+$ indicated a pattern similar to that of CD8$^+$ and CD25$^+$ (Figure 4a).

CD11c$^+$: CD11c$^+$ expression is similar to the expression of CD8$^+$, CD25$^+$, and WC1$^+$ (Figure 4b).

CD14 $^+$: The cells expressing CD14$^+$ were highly detected only in the normal young camels. The infected older camels had moderate expression (Figure 4c).

CX3CR1: The chemokine marker was only highly detected in the normal young camels. Its expression in the older camels was low (Figure 4d).

Madcam-1: The expression of Madcam-1 is similar to the expression of CD8$^+$,CD25$^+$, WC1$^+$, and CD11c$^+$ (Figure 4e).

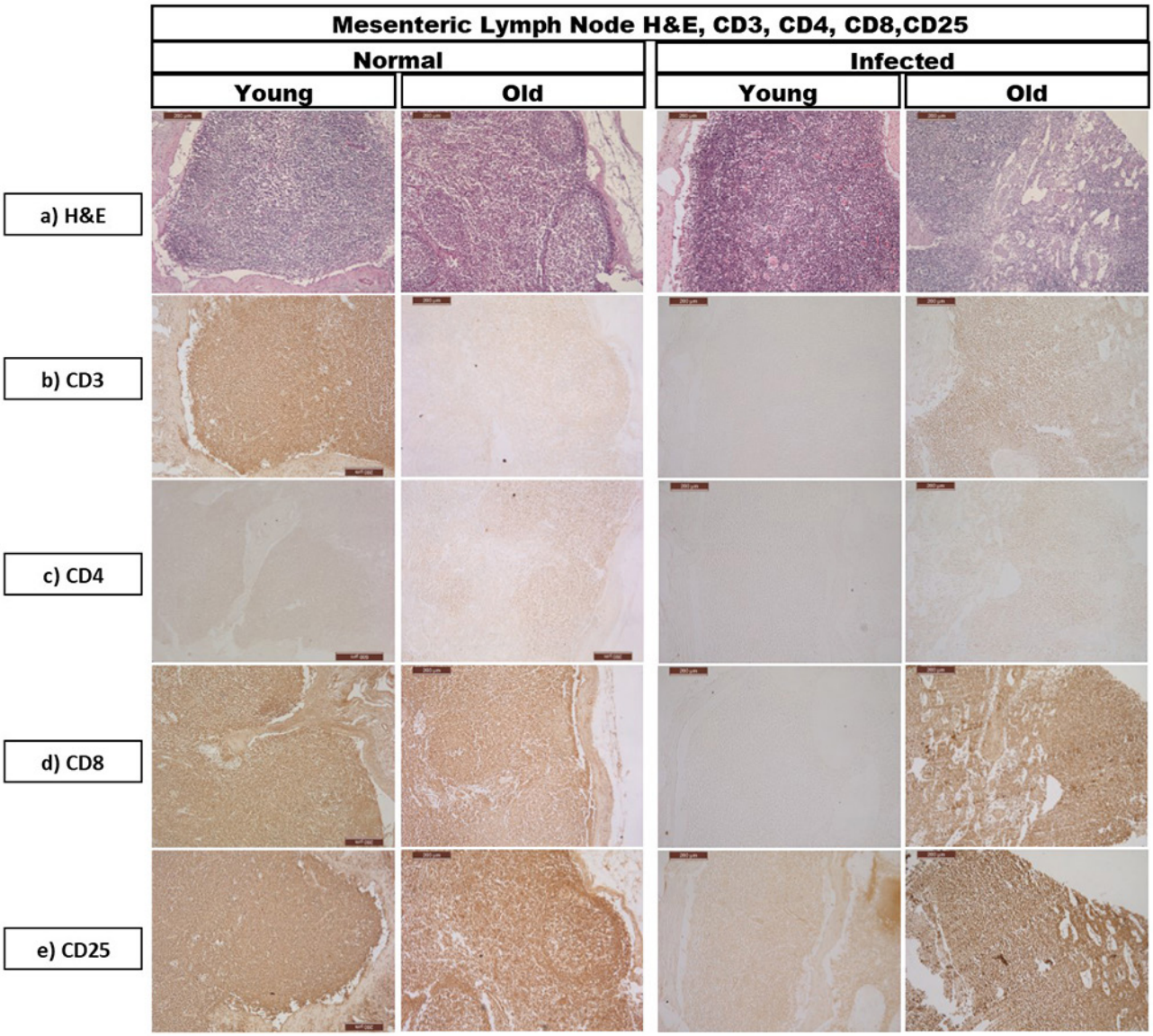

Figure 3. Histological sections from MLN of the young (5–10 years-old) and old (12–15 years-old) camels stained with (a) H&E; (b-e) Immunohistochemical detection of CD3, $CD4^+$, $CD8^+$& $CD25^+$, respectively. Notice $CD3^+$ was only detected on the cells in the MLN of the young normal and old infected camels (b). No expression of $CD4^+$in any of the camel's groups (c). The $CD8^+$ was extensively expressed on the cells of all groups except the MLN of the young infected camels (d). $CD25^+$ was extensively expressed on the cells of all groups except the MLN of the young infected camels (e). (Scale bar: 200µm).

The cellular distribution in the jejunum

H&E stain: The general structural features of the jejunum of normal and infected camels were posted for the exploration of any immunopositive cells within the jejunum in the subsequent sections (Figure 5a).

$CD3^+$: The prominent expression of $CD3^+$ was detected only in normal young and infected older camels (Figure 5b).

$CD4^+$: No $CD4^+$ expression was detected in any of the groups in the jejunum (Figure 5c).

$CD8^+$: No $CD8^+$ expression was detected in any of the groups in the jejunum (Figure 5d).

$CD25^+$: $CD25^+$ was highly expressed in the jejunum in all of the groups (Figure 5e).

$WC1^+$: The γδ cells expressing $WC1^+$ were high in all groups. The expression was concentrated mainly around the blood vessels of the submucosal tissues (Figure 6a).

$CD11c^+$: $CD11c^+$ was highly expressed in the jejunum of all of the groups except for the normal young camels (Figure 6b).

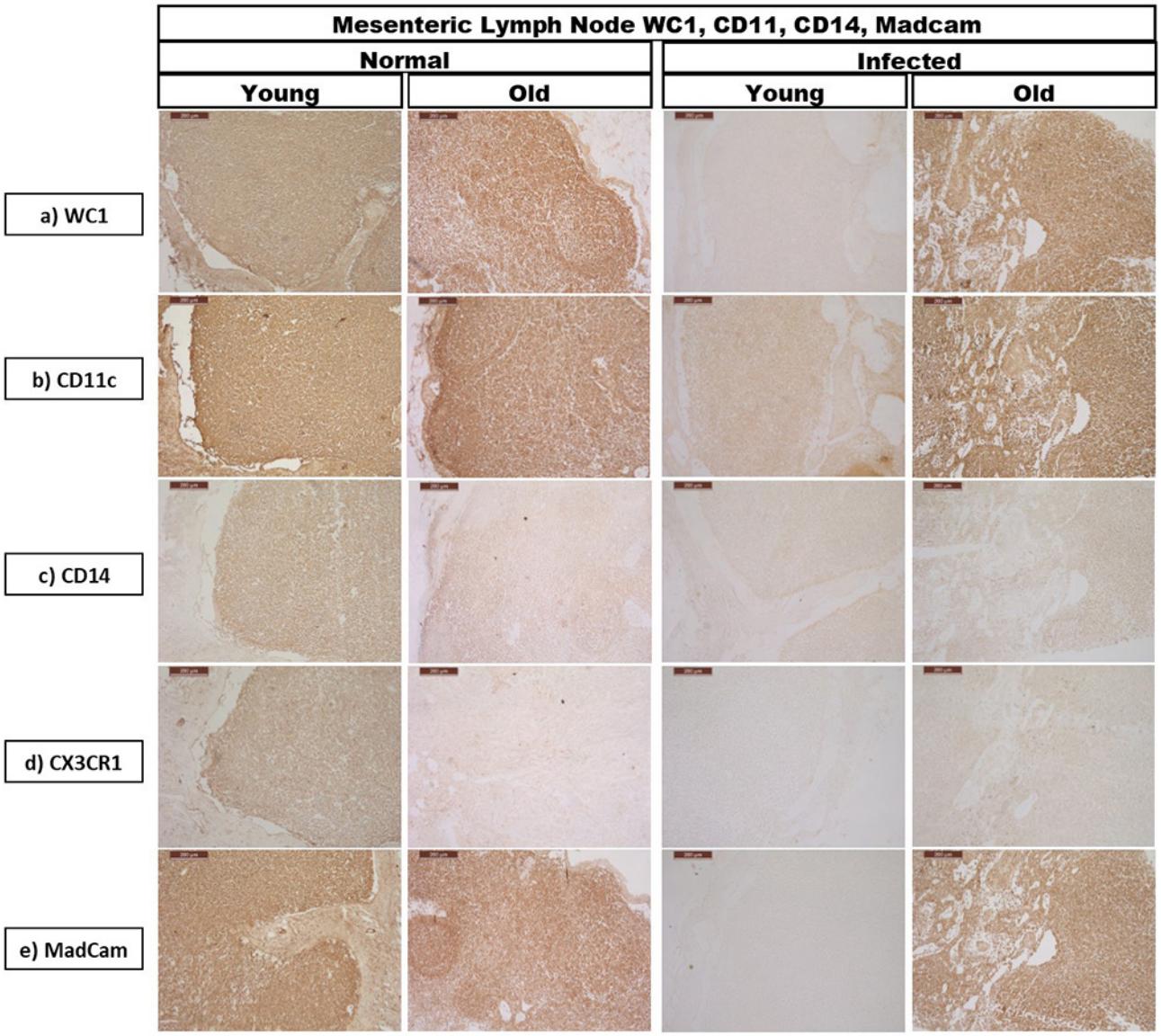

Figure 4. (a-e) Immunohistochemical detection of WC1$^+$, CX3CR1, CD11c$^+$, CD14$^+$ and Madcam-1 in MLN of different camel groups. Notice that the expressing WC1$^+$ was extensively expressed on the cells of all groups except the MLN of the young infected camels (a). Likewise, CD11c$^+$ was extensively expressed on the cells of all groups except the MLN of the young infected camels (b). CD14$^+$ expression was only detected in the young normal camels (c). CX3CR1 was detected only in the young normal camels (d). Madcam-1 was extensively expressed on the cells of all groups except the MLN of the young infected camels (e). (Scale bar: 200µm).

CD14 $^+$: The expression of the CD14$^+$ marker was only detected in the jejunum of the infected groups; the expression was high in the infected young camels (Figure 6c).

CX3CR1: Its expression was moderate to low in all of the camel groups. The CX3CR1 expression was mainly prominent around the epithelial tissues of villi (Figure 6d).

Madcam-1: The jejunum of all of the groups indicated high Madcam-1 expression except for the normal older camels (Figure 6e).

The cellular distribution in SML

H&E stain: The general structural features of the SML of normal and infected camels were posted for the exploration of any immunopositive cells within the SML in the subsequent sections (Figure 7a).

CD3$^+$, CD4$^+$, CD8$^+$: There was no expression for the markers CD3$^+$, CD4$^+$, or CD8$^+$(Figures 7b–7d).

CD25$^+$: CD25$^+$ was highly expressed in the SML of normal young and infected older camels (Figure 7e).

WC1$^+$: The gδ cells expressed WC1$^+$only in the normal young camels. The low expression was concentrated mainly around the follicles (Figure 8a).

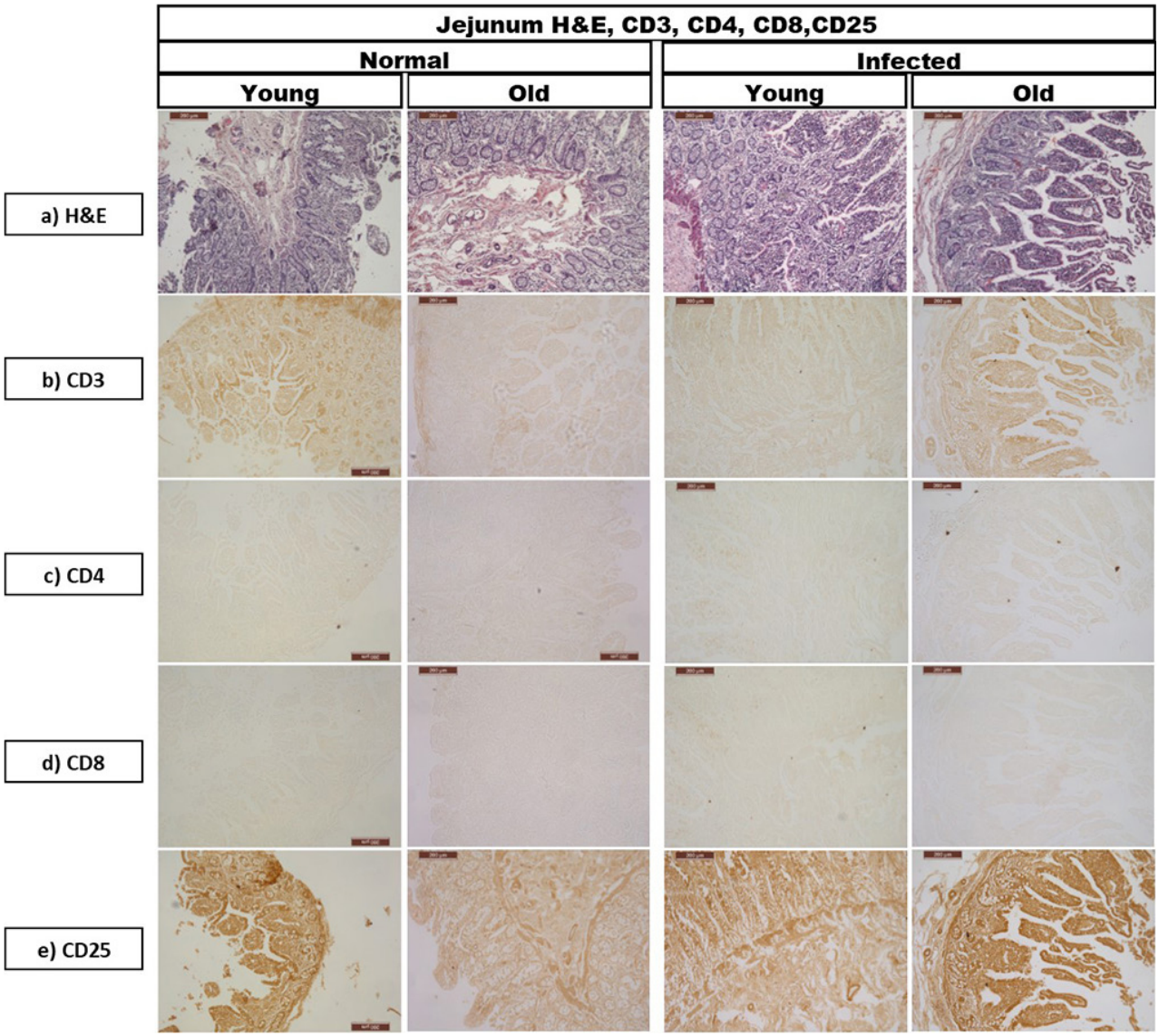

Figure 5. Histological sections from jejunum of the young (5–10 years-old) and old (12–15 years-old) camels stained with (a) H&E; (b-e) Immunohistochemical detection of $CD3^+$, $CD4^+$, $CD8^+$& $CD25^+$ respectively. Prominent expression of $CD3^+$ was detected only in young normal and old infected camels (b). No $CD4^+$ expression was detected in all of the groups in jejunum (c). No $CD8^+$ expression was detected in all of the groups in jejunum (d). $CD25^+$ was extensively expressed in the jejunum of all of the camel's groups (e). (Scale bar: 200μm).

$CD11c^+$: $CD11c^+$ was highly detected in the SML of normal young and infected older camel groups (Figure 8b). Moderate $CD11c^+$ expression levels were detected in infected young camels (Figure 8b).

$CD14^+$: No or low $CD14^+$ expression was seen in all of the groups (Figure 8c).

CX3CR1: Its expression was moderate only in the normal young camels (Figure 8d).

Madcam-1: No expression was detected in any of the groups (Figure 8e).

4. Discussion

The small intestine is the major residence of the MAP infection, primarily in the ileum and MLN [36,37]. Experimental studies have shown that the bacteria gain access to the small intestine either through the invasion of LP via microfold cells (M cells) or through the enterocytes [12].

The mucosal adaptive immunity of a normal small intestine is dominated by antiinflammatory responses [38,39]. The tolerance that plays a crucial role in maintaining

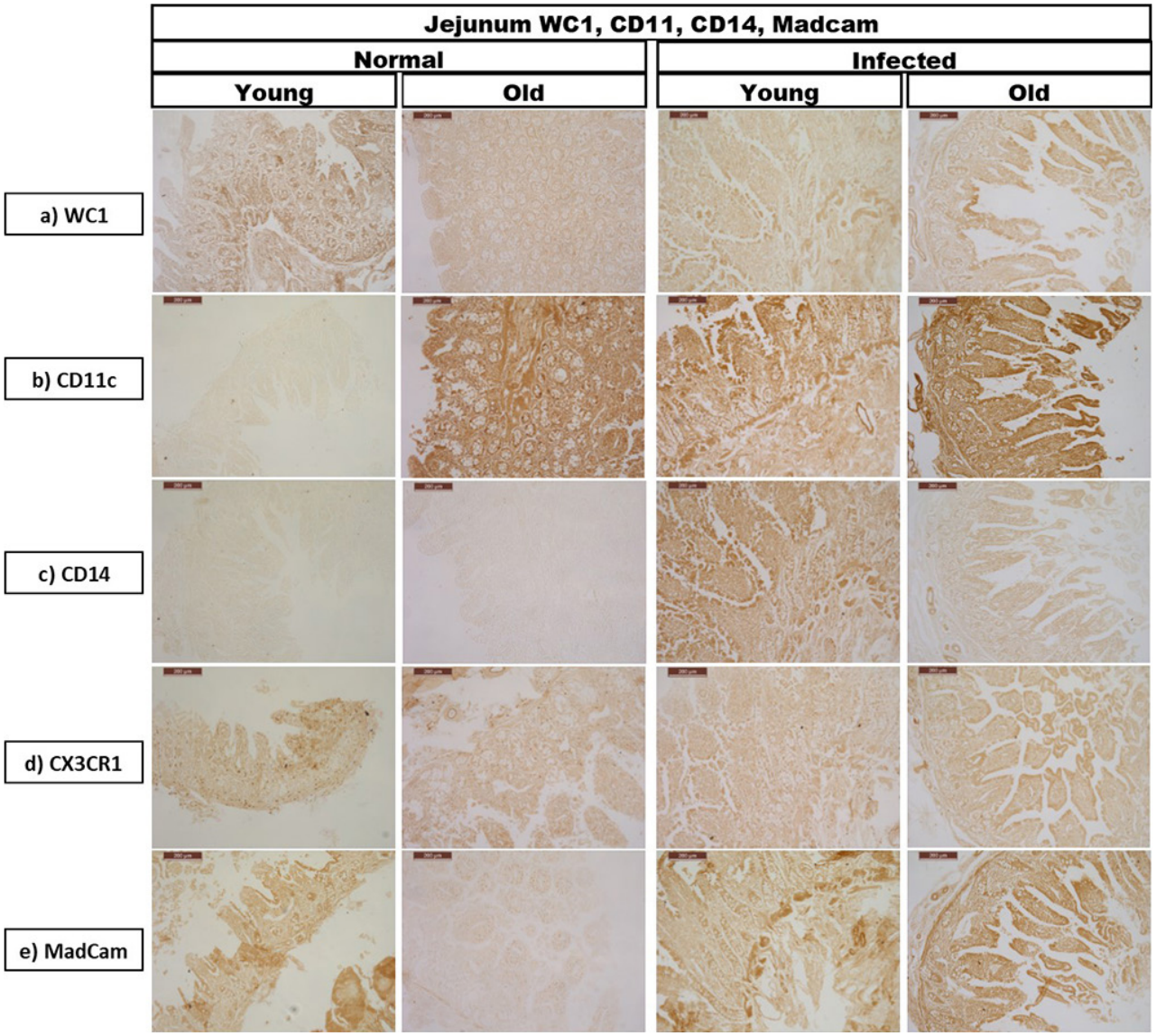

Figure 6: (a-e) Immunohistochemical detection of WC1$^+$, CX3CR1, CD11c$^+$, CD14$^+$ and Madcam-1 in the jejunum of different camel groups. WC1$^+$ were abundant in all camels' groups. The expression was concentrated mainly around the blood vessels of the submucosal tissues (a). CD11c$^+$ was detected extensively in all groups except the young normal camels (b). The expression of the CD14$^+$ was only detected in the infected groups specifically in the young infected camels (c). CX3CR1 expression was medium to low in all camel groups, mainly around the epithelial tissues of villi (d). Madcam-1 extensively expressed in all camel groups except the old normal camels (e). (Scale bar: 200μm).

intestinal homeostasis is due to the heavy presence of intestinal microbiota [39]. The intestinal homeostasis is achieved by a dense accumulation of special macrophage phenotypes characterized by the absence of CD14 and toll-like receptor-4 (TLR4) in the subepithelial LP. In addition to macrophages, intestinal homeostasis is also maintained by the presence of a high level of the CD4$^+$–CD25$^+$–Treg T-cells subset. These cells have antiinflammatory activity through the heavy production of transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) [38]. Failure of the tolerance will result in the enhancement of the expression of costimulatory markers like CD40, CD80, and CD86 and the expression of the proinflammatory marker CD14$^+$ [38].

The overall results clearly indicated that the majority of the markers were highly expressed in the ileum and MLN of the older camels. The CD markers that have shown high expression in ileum of infected older camels were CD8$^+$, CD25$^+$, WC1$^+$, CD11c$^+$, CD14$^+$, CX3CR1, and Madcam-1; the expression in MLN of the same group was similar to

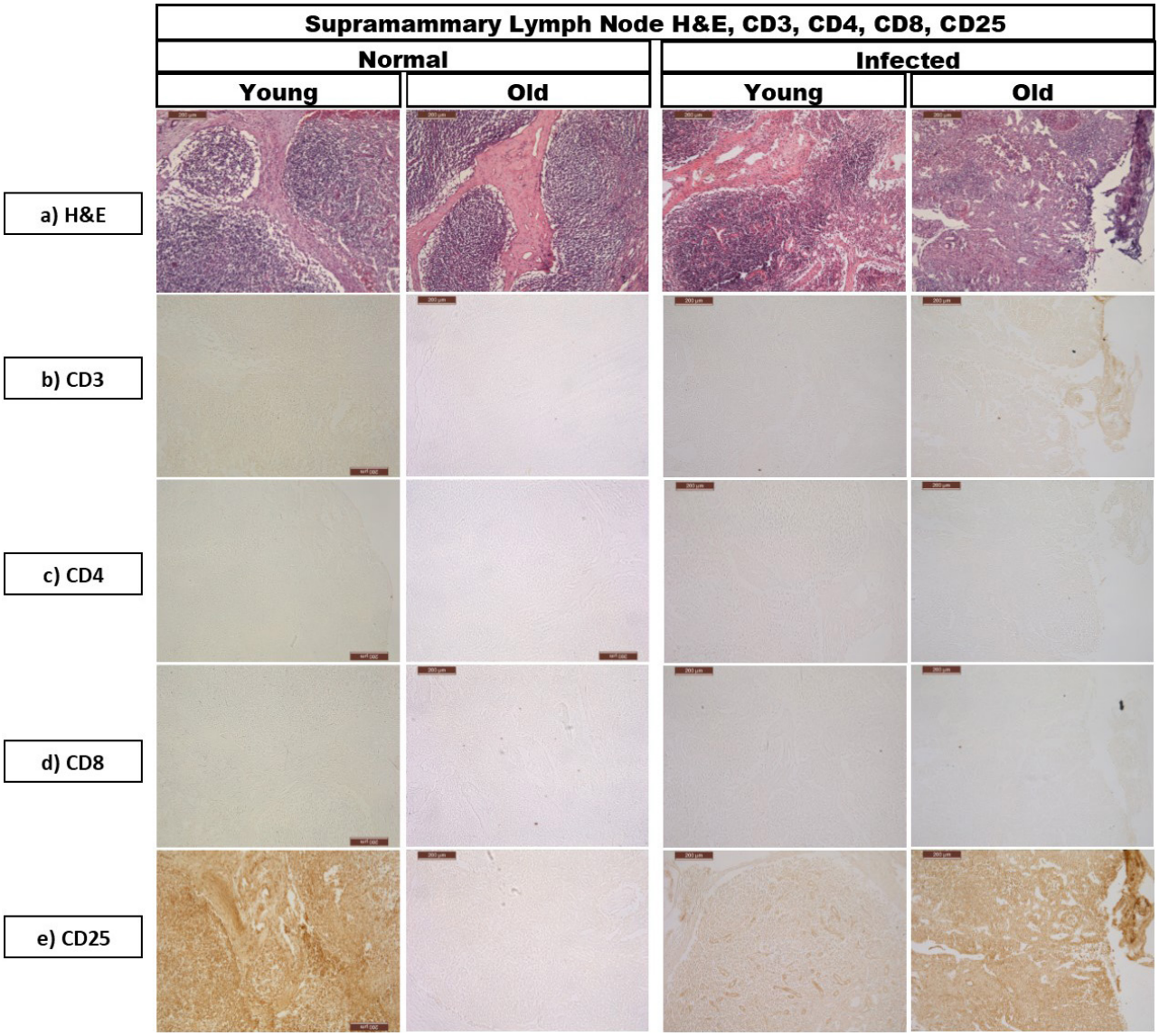

Figure 7. Histological sections from SML of the young (5–10 years-old) and old (12–15 years-old) camels stained with (a) H&E; (b-e) Immunohistochemical detection of CD3, CD4, CD8 &CD25 respectively. Notice there were no expressions for the markers in this tissue form all groups (b-e). Whereas, CD25+ was extensively expressed in the SML of young normal and old infected camels (e). (Scale bar: 200μm).

that of the ileum except for $CD14^+$ and CX3CR1. The high expression of the CD markers in the older camels is highly related to the chronic stage of the disease at this group [40]. The pathological studies of camel paratuberculosis have clearly indicated that the disease reaches its advanced stage in older camels [40].

The immunohistochemical staining of $CD4^+$ and $CD8^+$ cells revealed a significant accumulation of $CD8^+$ cells in the ileum and MLN of infected older camels, while $CD4^+$ cells were less expressed. These findings are in accordance with those of previous reports [14,15,17,41]. Navarro et al. [16] have reported that $CD8^+$ cells heavily accumulated in the LP of the goat intestine at the late stage of the natural infection [17]. Furthermore, Plattner et al. [40] has confirmed the dominance of the $CD4^+$ cells in the intestine of vaccinated calves but not that of infected animals [41]. Similar evidence was found by Charavaryamath et al. [14], in which their results indicated the evident accumulation of $CD8^+$ in the LP of the bovine ileal segment [15].

$CD25^+$ cells were expressed abundantly in the ileal and MLN tissues of all of the groups. In the intestine, the regulatory T-cells (Treg) were shown to play a major role in intestinal homeostasis [42]. Treg cells are a subset of $CD4^+$ T cells with coexpression of the $CD25^+$marker [40].

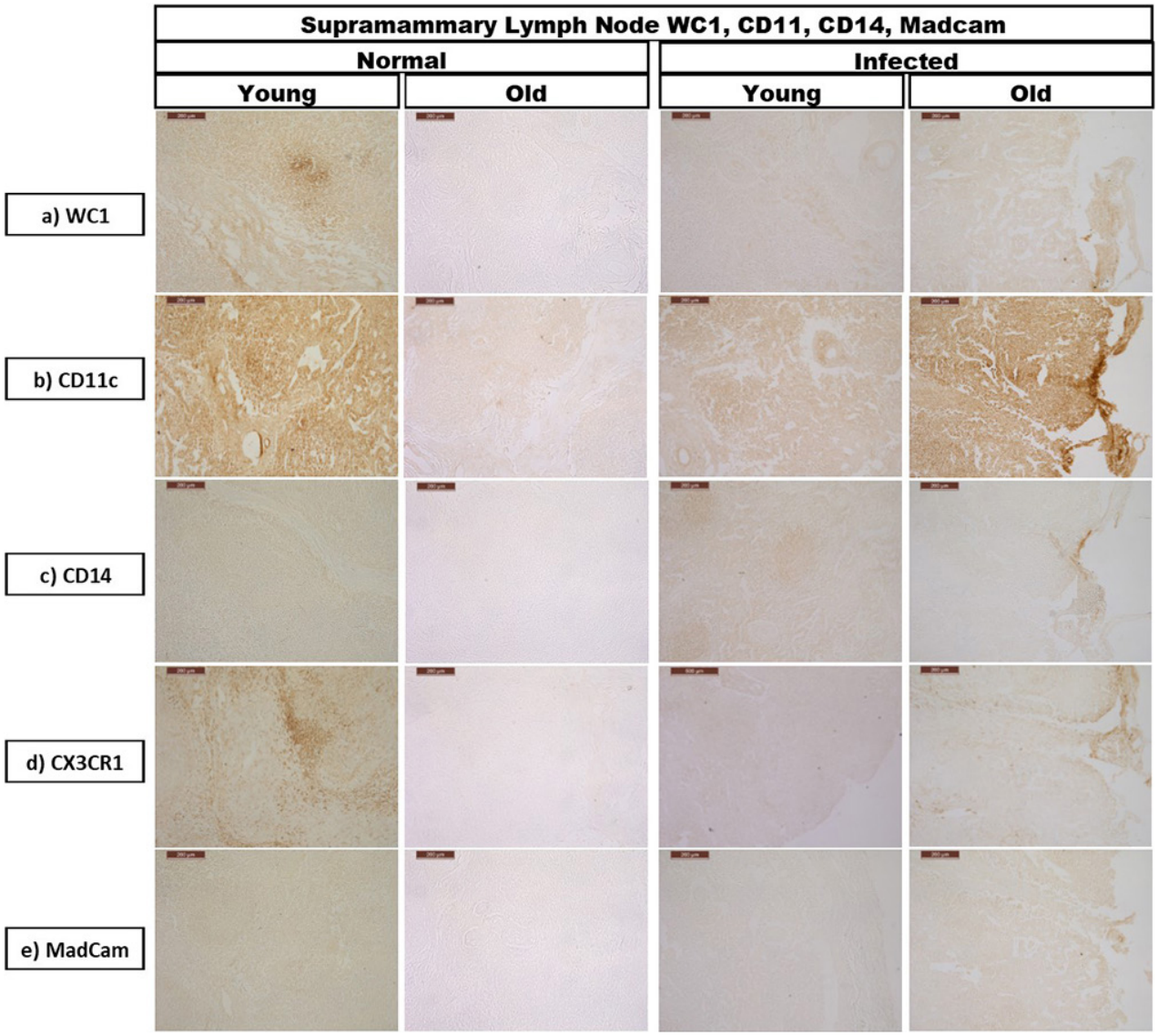

Figure 8. (a-e) Immunohistochemical detection of WC1$^+$, CX3CR1, CD11c$^+$, CD14$^+$ and Madcam-1 in SML of different camel groups. WC1$^+$ were only expressed in the young normal camels in which it is concentrated mainly around the follicles (a). CD11c$^+$ was detected extensively in the young normal and old infected camel groups, while medium expression level was detected in young infected camels (b). No or trivial CD14$^+$ expression was seen in all of the camel's groups (c). CX3CR1 expression was prominent in the young normal camels only (d). No expression of Madcam-1was detected in any of the camel's groups (e). (Scale bar: 200μm).

Treg cells exert tolerance by the copious production of IL-10 and TGF-β [42]. Interestingly, a study on the immune responses to MAP infection in cattle recorded high levels of Treg cells in the clinical stage of the disease [43]. In view of the low presence of CD4$^+$ cells in most of the groups' tissues, the high levels of CD25$^+$ cells are difficult to explain, particularly as the majority of the studies on MAP infection have confirmed the decline of the CD4$^+$ cells in the ileum and MLN.

γδ WC1$^+$ cells also showed heavy expression in the ileum. γδ WC1$^+$ cells are well known for their copious production of interferon-γ (IFN-γ) [44]. Experimental MAP infection studies have shown the accumulation of γδ WC1$^-$ cells in the ileal LP of infected calves at the early stage of the infection [41,45]. However, the WC1$^+$ γδ cells have also been recorded at high levels in the clinical stage of MAP infection [45]. γδ T-cells have been shown to exert suppressive regulation on CD4$^+$ T-cells in the intestine infected with MAP [43].

The results indicated high accumulation of myeloid cells, CD14$^+$ macrophages, and CD11c$^+$ DCs in the ileum of the infected older camels. These findings are compatible

with the findings reported by Charavaryamath et al. (2013) [14].

Innate immunity to the early presence of MAP is provoked by a wide range of pattern recognition receptors such as TLR, mannose receptors, and the complex of CD14/TLR4 [19]. CD14 is one of the major receptors in enhancing MAP phagocytosis by macrophages. Binding of the organism to CD14/TLR4 enhances the organism's internalization [46].

$CD11c^+$-DCs are considered important players in MAP pathogenesis in the intestine [15,16]. The DC cells that were retrieved from the granuloma expressed weak chemokine production and low expression of costimulatory receptors, which leads to downregulation of T-cell activation [16].

The immunohistochemical staining of the anti-CX3CR1 marker revealed a high presence of cells expressing this chemokine receptor in the ileum of the infected older camels. As an important player in preventing bowel inflammation in humans, the CX3CR1 macrophages have been a subject of extensive studies [27]. The CX3CR1 macrophages have a central role in the regulation of intestinal immune homeostasis, mucosal defense, and maintaining the barrier structure. They reduce the lumen microbial load and act as an antigen sampler through bidirectional shuttling through the intestinal barriers. The cells also have an important role in the exclusion of pathogens that have traversed to the intestinal epithelium [27]. The CX3CR1 macrophages are crucial in regulating intestinal tolerance, due to their extreme susceptibility to any changes in the gut environment [27].

Evidence for the accumulation of macrophages expressing the CX3CR1 chemokine receptor in mouse intestine with Crohn's disease was observed [46]. They expressed an adverse effect by producing a copious level of tumor necrosis factor-a (TNF-a) [46]. Crohn's disease is an inflammatory bowel disease; evidence has accumulated that incriminates MAP as one of the major inducers of this inflammatory reaction [47]. Hence, this link between Crohn's disease and CX3CR1 macrophages could justify the abundant expression of the CX3CR1 in the camel intestine. The intermediate CX3CR1 subtype, which has proinflammatory activity [27], could be involved in the inflammatory responses to the MAP infection.

The Madcam-1 expression was recorded extensively in the ileum, MLN, and jejunum. Immunostaining of the camel tissues indicated the expression of Madcam-1 in the alveolar tissue, supramammary and mesenteric lymph nodes, and Peyer's patches [25, 26]. Madcam-1 is a crucial molecule in the cell traffic to the intestine and MLN; therefore, it defines the nature of the cellular population involved in the innate and adaptive immunity of the intestine in both human and mouse. However, Madcam-1's role in camel intestinal immunity and MAP infection needs to be explored by taking into consideration the peculiarity of the camel as a ruminant species. Madcam-1's selective control of cellular trafficking to the intestine could be important in the recruitment of proinflammatory cellular populations during MAP infection. It has been shown that Madcam-1 is vital for the recruitment of LPMA-1–dependent $CD11c^+$–DCs to the intraepithelial tissue of the LP [22].

Therefore, the finding of high expression of the CX3CR1 macrophages and Madcam-1 in the camel intestine with MAP infection is novel and unprecedented. CX3CR1 and Madcam-1 expression could have a specific role in the immunopathological responses to MAP infection in the camel intestine which merits further exploration.

In conclusion, the overall results indicated the extensive presence of different lymphocyte subsets, DCs, and macrophages that have a vital role in orchestrating intestinal homeostasis and immune responses to MAP infection. The camel intestinal CD markers' expression in MAP infection was of high significance, mainly in the older camel group. The results overall were similar to those in reports of MAP infection in goat and cattle. However, high expression of Madcam-1 and CX3CR1 in the intestine of older camels clearly distinguishes camel from other ruminants. This novel finding suggests that the camel is closer to the monogastric animals in this aspect. Therefore, the expression of Madcam-1 could refer to its peculiar regulatory role in cellular trafficking in the camel intestine in both health and disease. Furthermore, the heavy presence of CX3CR1 macrophages could indicate a role in camel intestinal homeostasis. Hence, these novel findings clearly show the unconventional role of these molecules in the immunopathogenesis of MAP in the camel intestine. Therefore, further research on these aspects is highly recommended to highlight the significance of these molecules for MAP pathogenesis in the camel intestine.

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