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Immunohistochemical detection of CD14 and combined assessment with CD32B and CD68 for wound age estimation

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ABSTRACT

Estimation of wound age is a major topic of study for forensic pathologists, but few markers exist that can indicate a specific period 1–5 days postinfliction, and a method to estimate wound age with high accuracy has not yet been established. This study examined CD14 as such a marker in mouse skin wounds of different ages (0 min and 1, 2, 3, 5, 7, and 9 days) and in human subjects (group 1, 0–1 day; group 2, 1–5 days; group 3, >7 days) using Western blot analysis and/or immunohistochemical staining. In addition, we evaluated a combination of immunohistochemical markers in human skin wounds using transmembrane proteins, CD14, CD32B, and CD68, expressed on inflammatory cells. The expression of CD14 was detected only during 1–5 days postinfliction and, thus, the evaluation of CD14-expressing cells could specify wound age during 1–5 days postinfliction in mouse skin wounds. The ratio of samples assessed to be $CD14^+$ was significantly high in human skin wounds in group 2. Combined assessment using the three markers increased the specificity of diagnosis and shortened the range of wound age, compared with the assessment using a single marker. Our results indicate that CD14 may be a useful marker of wound age, 1–5 days postinfliction, and that combined assessment with CD14, CD32B, and CD68 may be a good method for the accurate estimation of wound age.

1. Introduction

Examination of wounds is one of the most important aspects in forensic practice. Forensic pathologists routinely estimate wound age based on appearance, such as the color of subcutaneous hemorrhage and scab formation, of the wound [1]. In addition, Berlin blue staining, which detects hemosiderin in subcutaneous tissues until approximately 7 days postinfliction, is conducted using conventional methods for wound age estimation [2]. However, it is difficult to accurately and objectively diagnose wound age at a stage as early as <7 days, particularly in 1–5 days postinfliction, using this method.

Several studies have conducted wound age estimation using biological substances [3–11]; however, only a few markers and methods were effective for high-accuracy wound age estimation in 1–5 days postinfliction. Therefore, identifying a marker and establishing a method for estimating wound age in 1–5 days postinfliction is important in forensic practice.

Cooper et al. [12] reported a portfolio of >1000 genes expressing across repair responses in neonatal mice using microarray analysis. Previously, we revealed that the mRNA expression of CD14, which is one of the genes exhibiting drastically changed time-course expression, reached peak levels at 12–24 h postinfliction in mouse skin [13]. In that study, our results indicated that the CD14 protein may be a useful marker in the 1–5 days postinfliction because there may be delayed protein expression following mRNA increase; this could be because of the additional time required to translate mRNA into protein, and protein expression may last much longer than that of the mRNA because of its high stability against degradation after death.

However, simultaneous detection of plural markers may be effective as a method to increase the accuracy of wound age estimation [3,10,11]. CD32B and CD68 are transmembrane proteins expressed on inflammatory cells similar to CD14. Therefore, we hypothesized that CD32B and CD68 may exhibit expression patterns similar to CD14.

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The objectives of this study were as follows: (1) to examine the expression of CD14 in mouse and human skin wounds with Western blotting and/or immunohistochemistry to evaluate the efficacy of CD14 as a marker for wound age estimation in 1–5 days postinfliction; (2) to conduct a preliminary study of whether the addition of markers, such as CD14-expressing cells, yielded more accurate estimation of wound age in mouse skin wounds; and (3) to investigate the time-course expression of CD32B and CD68 as well as CD14 to evaluate the effectiveness of combined assessment of wound age with these three markers by immunohistochemistry.

2. Materials and methods

2.1. Animal experiments

Pathogen-free 6-week-old male BALB/c mice purchased from SLC, Inc. (Shizuoka, Japan) were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and their dorsa were shaved. Furthermore, six full-thickness wounds 4 mm in diameter were resected from the dorsum of each mouse using biopsy punch. Each mouse was housed in a sterilized cage and given autoclaved food and redistilled water to prevent bacterial infection. The mice in the study groups ($n = 5$ for all groups except the 5–days group; $n = 4$ in the 5–days group) were euthanized at different time points of 0 min, 1, 2, 3, 5, 7, and 9 days after wound infliction. The entire wound area was harvested and stored at −80 °C until analysis. Skin tissues at time point of 0 min were examined as the control. The Animal Care Committee of Nagasaki University approved this research protocol (Approval No. 1410211181).

2.2. Human skin wound specimens

Human skin wound specimens [$n = 97$ from 44 individuals, age 16 days to 86 years (mean, 56.2 years)] of different postinfliction intervals, ranging from a few minutes to 30 days, were collected at forensic autopsies, and the postmortem interval till specimen collection was <3 days for all specimens. The wound specimens were classified into three groups according to wound age as follows: group 1 (0–1 day; $n = 38$), group 2 (1–5 days; $n = 50$), and group 3 (>7 days; $n = 9$). Samples of uninjured skin from the same individuals were collected as the control. The Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (Medical Course) approved this research protocol (Approval No. 15011660).

2.3. Antibodies

The following monoclonal or polyclonal antibodies (mAbs or pAbs) were used in this study: rabbit anti-CD14 pAbs (ab106285, Abcam, Cambridge, UK), rat anti-Ly6G6C mAbs (ab2557, Abcam), rat anti-F4/80 mAbs (ab6640, Abcam), rabbit anti-CD32B mAbs (ab45143, Abcam), mouse anti-CD68 mAbs (ab955, Abcam), goat anti-mouse CD14 pAbs (AF982, R&D Systems, Inc., Minneapolis, USA), rabbit anti-beta Tubulin pAbs (ab6046, Abcam), Histofine® Simple Stain Mouse Max-PO (R), Histofine® Simple Stain Max-PO (R), Histofine® Simple Stain Max-PO (M) (Nichirei Biosciences Inc., Tokyo, Japan), Alexa Fluor® 488 goat anti-rabbit IgG (H + L) antibody, Alexa Fluor® 568 goat anti-rat IgG (H + L) antibody (Thermo Fisher Scientific Inc., MA, USA), Secondary Antibody Solutions Alk-Phos. Conjugated (Anti-Rabbit), and Secondary Antibody Solutions Alk-Phos. Conjugated (Anti-Goat) (Thermo Fisher Scientific Inc.).

2.4. Western Blot analysis in mouse skin wounds

Skin tissues of mice were homogenized with a lysis buffer (50 mM Tris–HCl, pH 8.0, 2% SDS, 10 mM DTT) containing Phosphatase Inhibitor Cocktail and Protease Inhibitor Cocktail (Nacalai Tesque, Inc., Kyoto, Japan). After the protein concentration of all of the fractions was determined with a Bio-Rad protein assay (Bio-Rad Laboratories, California, USA), equal amount of proteins were electrophoresed on NuPAGE® Novex 4–12% Bis–Tris Gel (Thermo Fisher Scientific Inc.). Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane under 20 V for 7 min with iBlot™ (Thermo Fisher Scientific Inc.), and the membranes were treated with a PVDF Blocking Reagent from Can Get Signal® (TOYOBO CO., LTD., Osaka, Japan) for 30 min at room temperature and then incubated overnight at 4 °C with goat anti-mouse CD14 pAbs diluted to a 1:1000 concentration. β-Tubulin was used as an internal control. For detection, the membranes were incubated with a second antibody-solution – conjugated alkaline phosphatase at a 1:2000 dilution, and the blots were allowed to react with Novex® AP Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). After imaging with LAS-3000 mini (FUJIFILM, Tokyo, Japan), Quantity One (Bio-Rad Laboratories) was used to analyze the average density values.

2.5. Immunohistochemical analysis in mouse skin wounds

The wound tissue specimens of mouse were fixed in 4% paraformaldehyde with phosphate-buffered saline (PBS), embedded in paraffin, and cut into 3-μm sections with a microtome. Deparaffinized sections were irradiated with microwaves two times for 5 min in Dako Target Retrieval Solution, pH 9 (Agilent Technologies, California, USA). The sections were immersed in an endogenous peroxidase blocking reagent (0.3% H_2O_2, 0.2% phenylhydrazine, 1 mM PBS) for 30 min at room temperature, treated with Dako Protein Block Serum-Free Ready-to-use (Agilent Technologies) for 1 h at room temperature, and incubated overnight with rabbit anti-CD14 pAbs (dilution, 1:500) at 4 °C. Subsequently, the sections were reacted with Histofine® Simple Stain Mouse Max-PO (R) for 30 min and stained with a coloring reagent (50 mM Tris–HCl buffer, pH 7.6, 0.02% 3, 3′-diaminobenzidine, 0.006% H_2O_2) for 3 min at room temperature. Nuclear staining was performed by incubation with hematoxylin for 10 min at room temperature.

2.6. Double-color immunofluorescence analysis in mouse skin wounds

Double-color immunofluorescence analysis was conducted to determine the types of CD14-expressing cells during skin wound healing in the mouse. Deparaffinized sections were treated as described earlier with irradiation and endogenous peroxidase blocking. The sections were further incubated in a mixture of rabbit anti-CD14 pAbs (dilution 1:500) and rat anti-F4/80 mAbs (macrophage marker; dilution 1:500) or rabbit anti-CD14 pAbs (dilution 1:500) and rat anti-Ly6G6C mAbs (neutrophil marker; dilution 1:500) at 4 °C overnight. After incubation with a mixture of Alexa Fluor® 488 goat anti-rabbit IgG (H + L) antibody for rabbit anti-CD14 pAbs and Alexa Fluor® 568 goat anti-rat IgG (H + L) antibody for rat anti-F4/80 mAbs or rat anti-Ly6G6C mAbs for 1 h at room temperature, the sections were mounted with a VECTASHIELD Mounting Medium with DAPI (VECTOR LABORATORIES, INC. California, USA) and observed under a fluorescence microscope.

2.7. Immunohistochemical analysis in human skin wounds

Human wound specimens were fixed in 10% formaldehyde with PBS, embedded in paraffin, and then sliced into 3-μm sections with a microtome. Sections deparaffinized for the detection of CD14 were irradiated with microwaves two times for 5 min in Dako

Target Retrieval Solution (pH 9), and the sections deparaffinized for the detection of CD32B and CD68 were treated with Dako Proteinase K Ready-to-use (Agilent Technologies) for 6 min for antigen activation. These sections were treated as described earlier with endogenous peroxidase blocking followed by treatment with Dako Protein Block Serum-Free Ready-to-Use for 1 h at room temperature; they were then incubated overnight with rabbit anti-CD14 pAbs (dilution 1:500), rabbit anti-CD32B mAbs (dilution 1:400), and mouse anti-CD68 mAbs (dilution 1:400) at 4 °C. The sections were reacted with Histofine® Simple Stain Max-PO for rabbit or mouse immunoglobulin for 30 min and stained with 3,3′-diaminobenzidine for 3 min at room temperature after washing. Nuclear staining was conducted by incubation with hematoxylin for 10 min at room temperature.

2.8. Morphometric analysis in human skin wounds

In each section, four microscopic fields were randomly selected with X40 magnification. Ratios of markers (numbers of CD14-, CD32B-, or CD68- positive cells/the total number of infiltrating inflammatory cells) were calculated in four fields, and the average of these was recorded. The specimen was considered positive if at least 10% positivity was observed and negative if positivity was <10%.

2.9. Statistical analysis

2.9.1. Mouse skin wounds

A Mann–Whitney test was used for statistical evaluation of Western blotting results and $p < 0.05$ was considered to be statistically significant.

2.9.2. Human skin wounds

Fisher's exact probability test was used for statistical evaluation of the positive ratio between groups, with $p < 0.05$ considered to be statistically significant.

3. Results

3.1. Mouse skin wounds

3.1.1. Transition of CD14 expression at different post-traumatic intervals with Western Blotting

Expression of CD14 significantly increased on day 1 compared with the control (0 min) and days 7 and peaked at days 2–5 with a significant increase from that at 0 min and days 7 and 9 (levels decreased nearly to those in controls; Fig. 1a and b).

3.1.2. Immunohistochemical analysis of CD14

In the control group, there were no CD14-positive cells (Fig. 2a). CD14-positive cells gathered under the crust and surrounding skin on day 1 postinfliction (Fig. 2b). A large number of CD14-positive cells were observed with wide distribution around the wound on days 2–5 postinfliction (Fig. 2c–e) and were almost absent by days 7 and 9 postinfliction (Fig. 2f and g).

3.1.3. Berlin blue staining

Positive cells were observed at the wound site on days 7 and 9 postinfliction, although they were not detected in the control (0 min) or on days 1–5 (data not shown).

3.1.4. Double-color immunofluorescence analysis

CD14-expressing cells were immunostained with anti-Ly6G6C mAbs (for neutrophils) on days 1–3 postinfliction, whereas they were not detected on days 5 after wound infliction (Fig. 3). On the other hand, CD14-expressing cells were immunostained with anti-F4/80 mAbs (for macrophages) on days 2–5 postinfliction, although they were not detected on day 1 postinfliction (Fig. 4).

3.2. Human skin wounds

3.2.1. Immunohistochemical analysis of CD14, CD32B, and CD68

Representative staining images of CD14, CD32B, and CD68 are shown in Fig. 5. Positive stainings of CD14, CD32B, and CD68 were detected with a low intensity in the epidermis in uninjured skin specimens (Fig. 5a, d, and f). Expression of CD14 was observed on polymorphonuclear cells, considered to be neutrophils, on day 1 postinfliction (Fig. 5b) and observed on round-shaped mononuclear cells, considered to be macrophages, by days 5 postinfliction (Fig. 5c). Expressions of CD32B and CD68 were observed on round-shaped mononuclear cells on days 5 and 7 respectively (Fig. 5e and g).

3.2.2. Morphometrical analysis

The ratio of samples showing $CD14^+$, $CD32B^+$, and $CD68^+$ expression were calculated in each group as described in the following text.

3.2.2.1. $CD14^+$. As shown in Fig. 6a, the $CD14^+$ ratio in group 2 (100%; 50/50 samples) was significantly higher than in group 1 (7.9%; 3/38 samples) and group 3 (33.3%; 3/9 samples). $CD14^+$

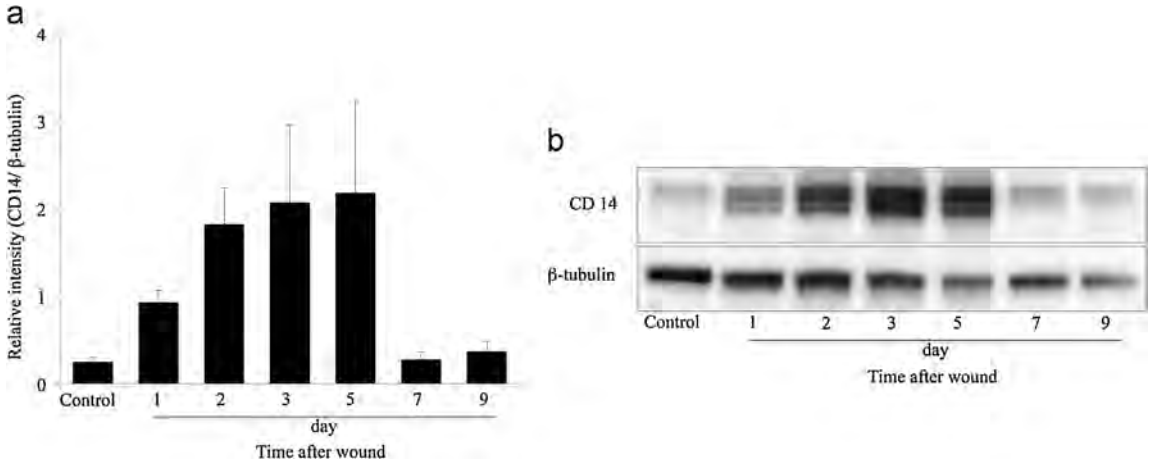

Fig. 1. Time-course expression of CD14 by Western blotting at different intervals postinfliction. (a) Relative intensity of CD14 expression to β-tubulin. Data are mean ± SEM. (b) Representative electrophoretic patterns of CD14 and β-tubulin.

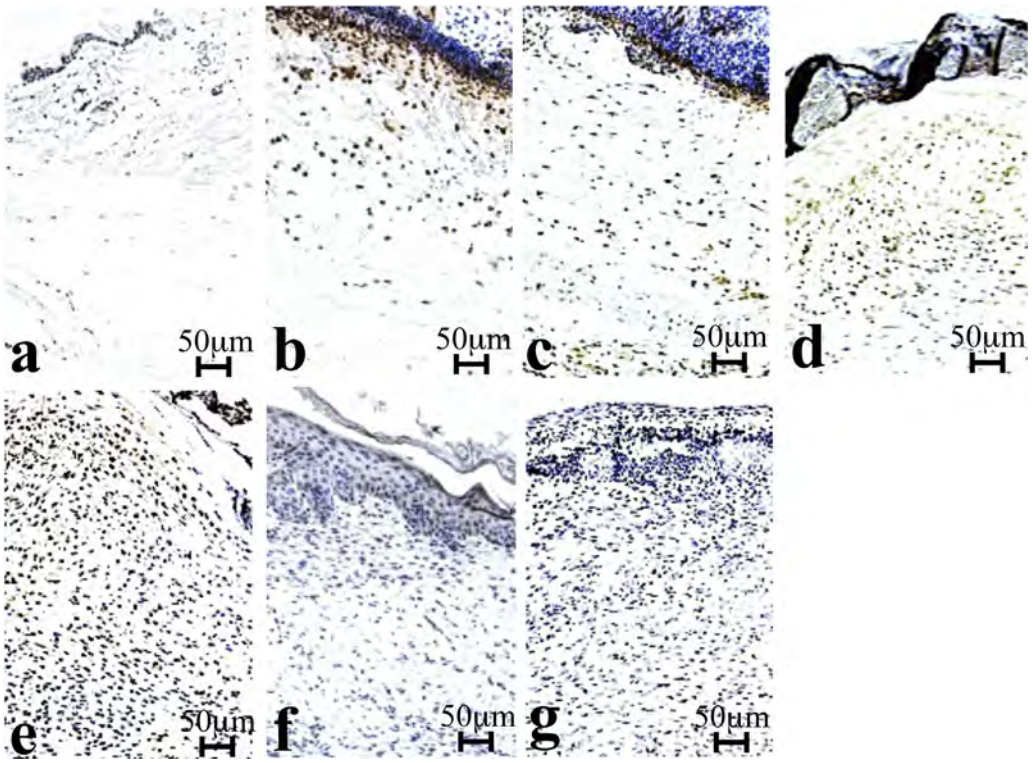

Fig. 2. Immunohistochemical analysis of CD14 expression in mouse skin during wound healing (postinfliction duration: a, 0 min; b, day 1; c, days 2; d, days 3; e, days 5; f, days 7; and g, days 9).

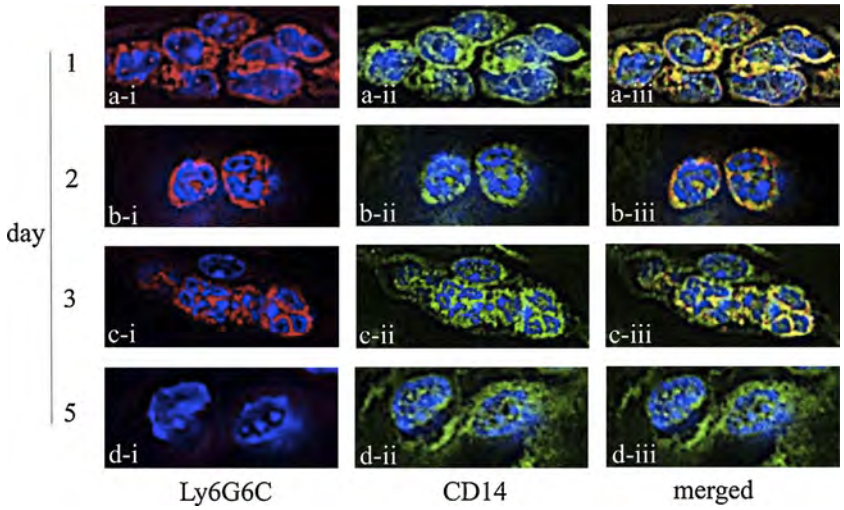

Fig. 3. Double immunofluorescence analysis of CD14 and Ly6G6C at days 1, 2, 3, and 5 postinfliction. The samples were immunostained with anti-Ly6G6C (i, red) and anti-CD14 (ii, green). Nuclei were counterstained with DAPI (i and ii, blue). Signals of Ly6G6C and CD14 were digitally merged in panel (iii) (original magnification ×500). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

represented wound ages of 1–5 days with a sensitivity of 100% and specificity of 87.2% (Table 1a).

3.2.2.2. CD32B$^+$. As shown in Fig. 6b, the CD32B$^+$ ratio in group 2 (64.0%; 32/50 samples) was significantly higher than those in group 1 (28.9%; 11/38 samples) and group 3 (22.2%; 2/9 samples) and has a sensitivity of 64.0% and specificity of 72.3% in determining wound age in wounds 1–5 days postinfliction (Table 1b).

3.2.2.3. CD68$^+$. As shown in Fig. 6c, the CD68$^+$ ratios of group 2 (76.0%; 38/50 samples) and group 3 (88.9%; 8/9 samples) were significantly higher than that in group 1 (7.9%; 3/38 samples) and had sensitivity of 78.0% and specificity of 92.1% in identifying wound age in wounds of >1 day (Table 1c). However, when we tested the converse that CD68$^-$ represents wound age of <1 day, the sensitivity was 92.1% and specificity was 78.0%.

3.2.3. Combinations of assessment with three markers

We calculated the ratios of samples that indicate a combination of assessment (positive or negative) with three markers (CD14, CD32B, and CD68) in each group (Table 2).

3.2.3.1. CD14$^+$/CD32B$^+$/CD68$^-$. Samples indicating this combination only belonged to group 2, and when tested to determine

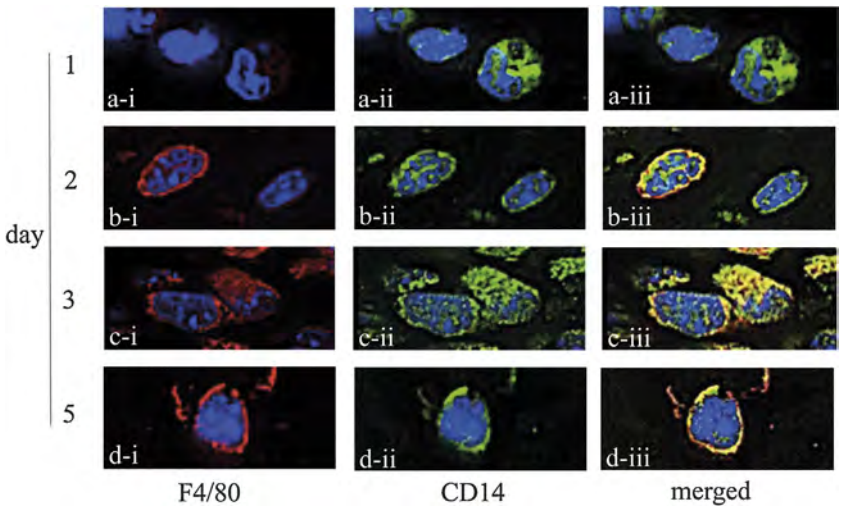

Fig. 4. Double immunofluorescence analysis of CD14 and F4/80 at days 1, 2, 3, and 5 postinfliction. The samples were immunostained with anti-F4/80 (i, red) and anti-CD14 (ii, green). Nuclei were counterstained with DAPI (i and ii, blue). Signals of F4/80 and CD14 were digitally merged in panel (iii) (original magnification ×500). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

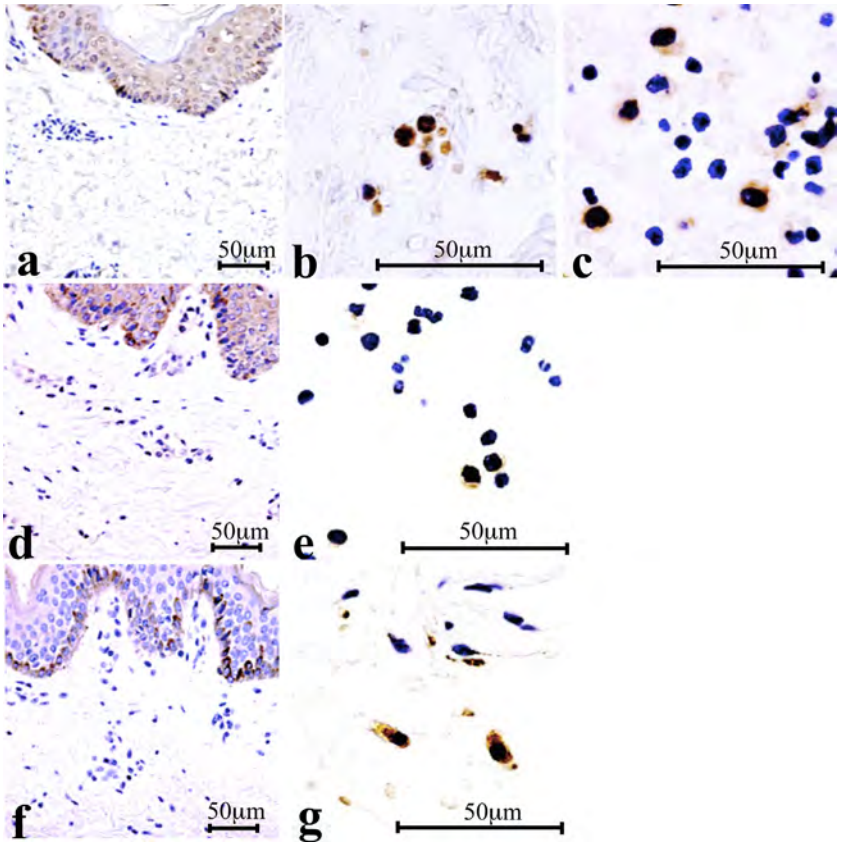

Fig. 5. Immunohistochemical staining of (a) CD14; (d) CD32B; and (f) CD68 in human uninjured skin and of CD14 (b and c, wound age of 1 and 5 days, respectively), CD32B (e, 5 days), and CD68 (g, 7 days) in human skin wounds.

wound age of 1–5 days, the sensitivity was 20.0% and specificity was 100% (Table 3a).

3.2.3.2. $CD14^{-}/CD32B^{-}/CD68^{+}$. Samples indicating this combination only belonged to group 3 and showed a sensitivity of 55.6% and specificity of 100% in determining wounds older than 7 days (Table 3b).

3.2.3.3. $CD14^{-}/CD32B^{-}/CD68^{-}$. Samples indicating this combination belonged to group 1 and showed sensitivity of 68.4% and specificity of 98.3% in identifying wounds <1 day old (Table 3c).

4. Discussion

The initial aim of this study was to identify a marker for wound age of between 1 and 5 days for application in forensic practice. We hypothesized that the CD14 protein could be a marker in the 1–5 days after wound infliction because the expression of CD14 mRNA in mouse skin was reported to show peak levels at 12–24 h postinfliction in a previous study [13].

CD14 is a 55-kDa glycosylphophatidylinositol-anchored protein expressed on the surface of monocytes, macrophages, and neutrophils; it is known to be a receptor for complexes of

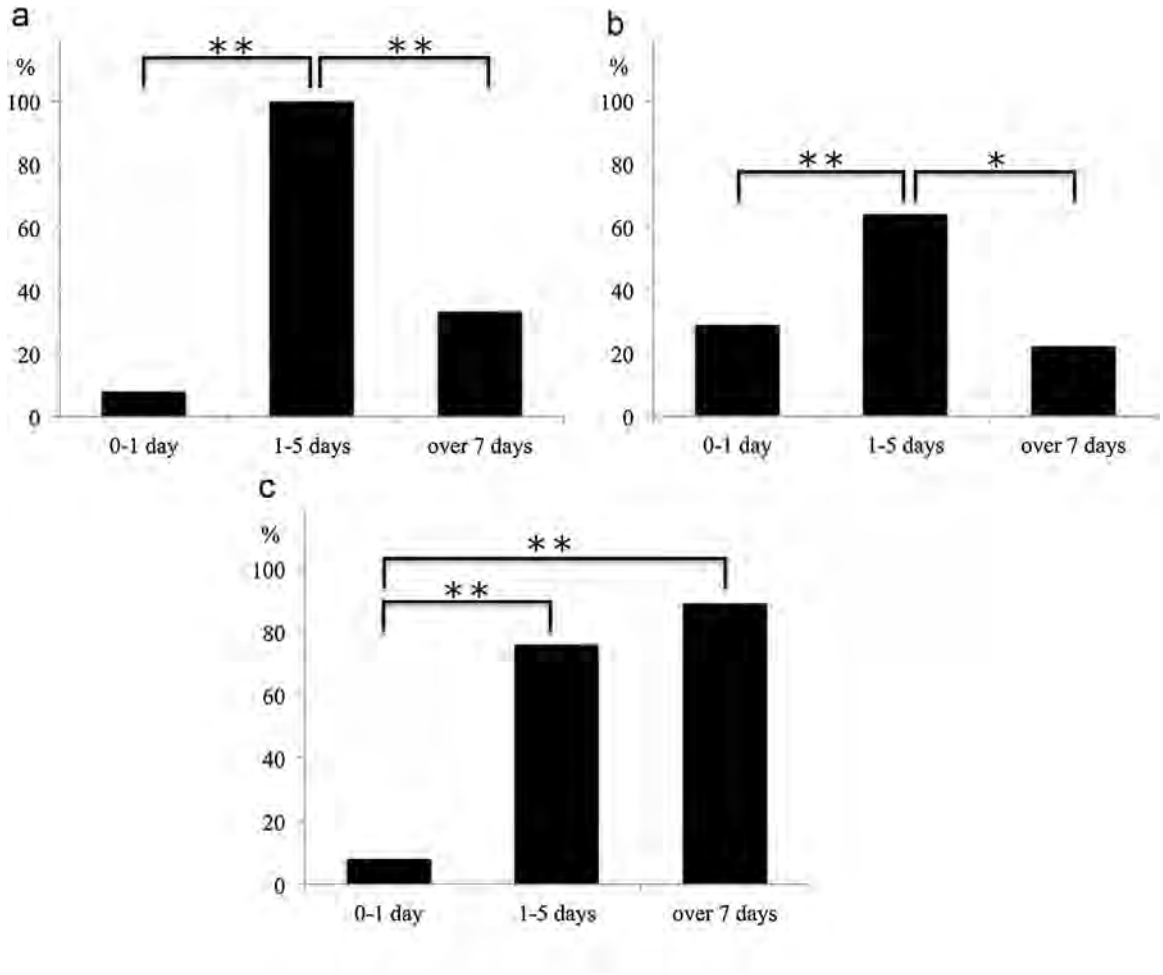

Fig. 6. The ratio of (a) $CD14^+$, (b) $CD32B^+$, and (c) $CD\ 68^+$ samples in each wound group. *, $p < 0.05$; **, $p < 0.01$.

Table 1
Sensitivity and specificity of immunohistochemistry with $CD14^+$, $CD32B^+$, and $CD68^+$ for determination of wound age.

Marker	group 2 (1–5 days)	groups 1 (0–1 day) and 3 (>7 days)
	n	*n*
(a)		
$CD14^+$	50	6
$CD14^-$	0	41
Total	50	47
	Sensitivity 100% (50/50)	Specificity 87.2% (41/47)
Marker	group 2 (1–5 days)	groups 1 (0–1 day) and 3 (>7 days)
	n	*n*
(b)		
$CD32B^+$	32	13
$CD32B^-$	18	34
Total	50	47
	Sensitivity 64.0% (32/50)	Specificity 72.3% (34/47)
Marker	groups 2 (1–5 days) and 3 (>7 days)	group 1 (0–1 days)
	n	*n*
(c)		
$CD68^+$	46	3
$CD68^-$	13	35
Total	59	38
	Sensitivity 78.0% (46/59)	Specificity 92.1% (35/38)

Table 2
Ratios in samples by combined assessment with three markers in each group.

Combinations of assessment			Ratio in each group (%)		
CD14	CD32B	CD68	group 1 (0–1 day)	group 2 (1–5 days)	group 3 (>7 days)
+	+	+	2.6	44.0	22.2
+	+	–	0	20.0	0
+	–	+	0	28.0	11.1
+	–	–	5.3	8.0	0
–	+	+	5.3	0	0
–	+	–	18.4	0	0
–	–	+	0	0	55.6
–	–	–	68.4	0	11.1
		Total	100	100	100

+; Positive, –; Negative.

lipopolysaccharide (LPS) and its binding protein [14,15]. It plays an important role in LPS-mediated induction of cytokines such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1, IL-6, and IL-8 [16–19]. Various studies revealed that CD14 is highly expressed in the bone marrow of patients with chronic myelomonocytic leukemia, in blood neutrophils in patients with Kawasaki disease, and in the serum of patients with severe burns [20–22]. However, to the best of our knowledge, there have been no reports on the expression of CD14 in human skin wounds from the viewpoint of wound age estimation.

Table 3
Sensitivity and specificity of wound age estimation with $CD14^+/CD32B^+/CD68^-$, $CD14^-/CD32B^-/CD68^+$, and $CD14^-/CD32B^-/CD68^-$.

Markers	group 2 (1–5 days)	groups 1 (0–1 day) and 3 (>7 days)
	n	*n*
(a)		
$CD14^+/CD32B^+/CD68^-$	10	0
Other combinations	40	47
Total	50	47
	Sensitivity 20.0% (10/50)	Specificity 100% (47/47)
Markers	group 3 (>7 days)	groups 1 (0–1 day) and 2 (1–5 days)
	n	*n*
(b)		
$CD14^-/CD32B^-/CD68^+$	5	0
Other combinations	4	88
Total	9	88
	Sensitivity 55.6% (5/9)	Specificity 100% (88/88)
Markers	group 1 (0–1 day)	groups 2 (1–5 days) and 3 (>7 days)
	n	*n*
(c)		
$CD14^-/CD32B^-/CD68^-$	26	1
Other combinations	12	58
Total	38	59
	Sensitivity 68.4% (26/38)	Specificity 98.3% (58/59)

In this study, we conducted Western blot analysis and immunohistochemical staining of CD14 in mouse skin wounds. First, Western blot analysis revealed that CD14 expression in days 2–5 postinfliction significantly increased compared with that at 0 min and days 7 and 9 as did that on day 1 compared with that at 0 min and days 7. Second, immunohistochemical analysis showed that the accumulation of CD14-positive cells was only detected on days 1–5, whereas positive cells of Berlin blue staining were observed in mouse skin wounds at days 7 and 9, and there were no staining of cells at 0 min and from days 1–5. Thus, the expressions of CD14 at the wound site could indicate a wound age of 1–5 days, although this could not be determined based on Berlin blue staining.

Subsequently, we also conducted immunohistochemical staining for CD14 in human skin wounds based on the results from mouse experiments. Suggesting the morphology of expressing cells and expression period as shown in Figs. 5e and g and 6b and c, we speculate that the expressing cells of CD32B might be neutrophil and macrophage and those of CD68 could be almost macrophage, although we realise that further study will need to determine it. The positive ratio of CD14 in group 2 significantly exceeded those of groups 1 and 3. This finding indicated that $CD14^+$ could be a useful marker for detecting wound ages of 1–5 days in forensic practice. Moreover, CD14 expressions in all samples belonging to group 2 were positive. In other words, the sensitivity of diagnosis of wound age between 1 and 5 days was 100%. Our results suggested that the $CD14^-$ skin samples could be excluded from analysis of wound age of 1–5 days because of the high sensitivity of $CD14^+$. The ratio of negative samples belonging to groups 1 and 3 on days 0–1 or >7 days, indicated that the specificity of diagnosis of wound age between 1 and 5 days was 87.2%. Given the high sensitivity and specificity of $CD14^+$ in wounds, CD14 may have the high applicability as a wound estimation marker.

Beschorner et al. [23] reported that CD14 expression in perivascular spaces and the brain parenchyma increased within 1–2 days, reached a maximum level within 4–8 days, and remained elevated for weeks at the lesion and adjacent areas following traumatic brain injury in humans. Our findings similarly corresponded with the report on the kinetics of CD14 until they reached the maximum level; however, they did not replicate that described for the subsequent period. Therefore, we infer that the mechanism of CD14 expression in the skin may differ from that in the brain. We identified that CD14-positive cells in human skin wounds on day 1 postinfliction were polymorphonuclear cells, considered to be neutrophils, and those on days 5 were round-shaped mononuclear cells, considered to be macrophages. Thus, we assumed that the type identification of CD14-expressing cells may be useful to estimate wound age with greater accuracy.

We examined CD14-expressing cells in mouse skin wounds on days 1–5 postinfliction by double-color immunofluorescence microscopy. The results revealed that CD14 is expressed on neutrophils using anti-Ly6g6c on days 1–3 and on macrophages using F4/80 on days 2–5. These findings could indicate that the expression of CD14 on both neutrophils and macrophages could indicate wound age of 2–3 days and expression only on neutrophils or macrophages could indicate wound age of 1 day or 5 days, respectively. Therefore, the examination of CD14-expressing cell types narrows the period of wound age estimation. Moreover, simultaneous determination of two cell types may directly help to reveal wound age.

To apply the results from mouse skin wounds to forensic practice, we predicted that a method combining assessment with the markers selected by the similarity of expression period was better than examining CD14-expressing cells with double-color immunofluorescence analysis. The reason for this was that additional examination with different markers by the method of combined assessment should be used only as required. In addition, we could test the combination of more available markers compared with those used in the method examining CD14-expressing cells, so that the former method may enable improvement of the precision of wound age estimation in the future.

We examined the expression of CD32B and CD68 in human skin wounds and evaluated the utility of these protein expressions in determining wound age. CD32B is a low-affinity inhibitory FCγ receptor of immunoglobulin G that influences innate immunity. It is widely expressed on effector cells such as macrophages and neutrophils. In macrophages, CD32B cross-linking inhibits FCγ receptor-mediated phagocytosis and cytokine release. In neutrophils, cross-linking of activating FCγ receptors results in phagocytosis; superoxide production; and enhanced neutrophil adhesion, rolling, and migration, all of which are probably inhibited by ligating CD32B [24,25]. CD68 belongs to a family of lysosomal-associated membrane glycoproteins and is largely expressed in monocytes and macrophages; it is widely used as a macrophage marker. Although the biological function of CD68 has not been completely defined, it serves as a scavenger receptor for oxidized low-density lipoproteins and may also be involved in cell–cell interactions [26,27].

Positive ratio of CD32B in group 2 significantly exceeded the ratio in groups 1 and 3. This finding indicated that $CD32B^+$ could be a marker of wound age of 1–5 days. However, the sensitivity (64.0%) and the specificity (72.3%) were lower than that with $CD14^+$. Because the positive ratio of CD68 in groups 2 and 3 significantly exceeded that in group 1, $CD68^+$ could be a marker for wound age at least 1 day after wound infliction. Sensitivity (78.0%) and specificity (92.1%) were sufficiently high and were of the level required for use in forensic practice. The negative ratio of CD68 in group 1 significantly exceeded those in groups 2 and 3. This result showed that $CD68^-$ may be a marker of wound age of <1 day. High sensitivity (92.1%) and specificity (78.0%) based on the converse of $CD68^+$ prove the utility of $CD68^-$ as a marker.

Our results indicated that three proteins (CD14, CD32B, and CD68) could be useful as markers to estimate wound age and that their expression periods were closely associated. Furthermore, we

evaluated the utility of combined assessment using these three proteins in wound age determination. Samples showing a combination of $CD14^+/CD32B^+/CD68^-$ expression only belong to group 2. Thus, this combination indicates that the wound age may be between 1 and 5 days with high specificity (100%); however, the sensitivity (20.0%) of this combination was very low. Although $CD14^+$ could independently indicate wound age of 1–5 days, the supporting evidence by $CD32B^+/CD68^-$ raises the specificity to 100%, such that a definitive diagnosis could be made.

The combination of $CD14^-/CD32B^-/CD68^+$ was identified only in group 3. Therefore, this pattern indicates that the wound age may be at least 7 days. Because $CD68^+$ could independently reveal wound age of at least 1 day, the combination assay of the three markers narrowed the period of wound age estimation. In addition, the assay may be available for a definitive diagnosis of wounds of at least 7 days because of its high specificity (100%). More than two thirds of the samples in group 1 showed a pattern of $CD14^-/CD32B^-/CD68^-$ as did a few samples that belonged to group 3. Therefore, this combination would indicate wound age of <1 day as well as that shown by $CD68^-$; however, the specificity of the pattern (98.3%) is increased compared with that of only $CD68^-$ (78.0%). This combination may be useful to definitely diagnose wound age of <1 day.

Several reports have shown the utility of wound age estimation by using plural markers [2,11,12]; however, our method is definitively different from those using combined assessment with plural markers as a key to accurate estimation.

In conclusion, our study indicates that $CD14^+$ could be a useful marker of wound age 1–5 days postinfliction in forensic practice. Moreover, our results show that a method of combined assessment with our markers, CD32B and CD68, in addition to CD14 may improve the specificity of wound age estimation and narrow the range of wound age more than those with a method using a single marker. Future research efforts examining a suitable combination among various markers may enable direct determination of wound age, particularly the number of days postinfliction.

Conflict of interest

There are no conflicts of interest to declare.

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References

[1] F. Takabe, Vital reaction in skin wounds, Res. Pract. Forensic Med. 34 (1991) 1–33.
[2] P. Betz, Histological and enzyme histochemical parameters for the age estimation of human skin wounds, Int. J. Legal Med. 107 (2) (1994) 60–68.
[3] P. Betz, W. Eisenmenger, Morphometrical analysis of hemosiderin deposits in relation to wound age, Int. J. Legal Med. 108 (5) (1996) 262–264.
[4] J. Raekallio, Determination of the age of wounds by histochemical and biochemical methods, Forensic Sci. 1 (1) (1972) 3–16.
[5] T. Ohshima, Forensic wound examination, Forensic Sci. Int. 113 (1–3) (2000) 153–164.
[6] T. Kondo, T. Ohshima, R. Mori, D.W. Guan, K. Ohshima, W. Eisenmenger, Immunohistochemical detection of chemokines in human skin wounds and its application to wound age determination, Int. J. Legal Med. 116 (2) (2002) 87–91.
[7] T. Kondo, Timing of skin wounds, Legal Med. (Tokyo) 9 (2) (2007) 109–114, http://dx.doi.org/10.1016/j.legalmed.2006.11.009.
[8] J. Dressler, L. Bachmann, R. Koch, E. Müller, Estimation of wound age and vcam-1 in human skin, Int. J. Legal Med. 112 (3) (1999) 159–162.
[9] W. Grellner, Time-dependent immunohistochemical detection of proinflammatory cytokines (il-1beta, il-6, tnf-alpha) in human skin wounds, Forensic Sci. Int. 130 (2–3) (2002) 90–96.
[10] M. Takamiya, H. Biwasaka, K. Saigusa, N. Nakayashiki, Y. Aoki, Wound age estimation by simultaneous detection of 9 cytokines in human dermal wounds with a multiplex bead-based immunoassay: an estimative method using outsourced examinations, Legal Med. (Tokyo) 11 (4) (2009) 186–190, http://dx.doi.org/10.1016/j.legalmed.2009.03.010.
[11] F.R.W. van de Goot, H.I. Korkmaz, J. Fronczek, B.I. Witte, R. Visser, M.M.W. Ulrich, et al., A new method to determine wound age in early vital skin injuries: a probability scoring system using expression levels of fibronectin, cd62p and factor viii in wound hemorrhage, Forensic Sci. Int. 244 (2014) 128–135, http://dx.doi.org/10.1016/j.forsciint.2014.08.015.
[12] L. Cooper, C. Johnson, F. Burslem, P. Martin, Wound healing and inflammation genes revealed by array analysis of 'macrophageless' pu.1 null mice, Genome Biol. 6 (1) (2005) R5, http://dx.doi.org/10.1186/gb-2004-6-1-r5.
[13] S. Kagawa, A. Matsuo, Y. Yagi, K. Ikematsu, R. Tsuda, I. Nakasono, The time-course analysis of gene expression during wound healing in mouse skin, Legal Med. (Tokyo) 11 (2) (2009) 70–75, http://dx.doi.org/10.1016/j.legalmed.2008.09.004.
[14] T.L. Kielian, F. Blecha, Cd14 and other recognition molecules for lipopolysaccharide: a review, Immunopharmacology 29 (3) (1995) 187–205.
[15] S.D. Wright, R.A. Ramos, P.S. Tobias, R.J. Ulevitch, J.C. Mathison, Cd14, a receptor for complexes of lipopolysaccharide (lps) and lps binding protein, Science 249 (4975) (1990) 1431–1433.
[16] C. Couturier, G. Jahns, M.D. Kazatchkine, N. Haeffner-Cavaillon, Membrane molecules which trigger the production of interleukin-1 and tumor necrosis factor-alpha by lipopolysaccharide-stimulated human monocytes, Eur. J. Immunol. 22 (6) (1992) 1461–1466, http://dx.doi.org/10.1002/eji.1830220619.
[17] D. Heumann, P. Gallay, C. Barras, P. Zaech, R.J. Ulevitch, P.S. Tobias, et al., Control of lipopolysaccharide (lps) binding and lps-induced tumor necrosis factor secretion in human peripheral blood monocytes, J. Immunol. 148 (11) (1992) 3505–3512.
[18] T.R. Martin, J.C. Mathison, P.S. Tobias, D.J. Letúrcq, A.M. Moriarty, R.J. Maunder, et al., Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. implications for cytokine production in normal and injured lungs, J. Clin. Invest. 90 (6) (1992) 2209–2219, http://dx.doi.org/10.1172/JCI116106.
[19] M.A. Dentener, V. Bazil, E.J. Von Asmuth, M. Ceska, W.A. Buurman, Involvement of cd14 in lipopolysaccharide-induced tumor necrosis factor-alpha, il-6 and il-8 release by human monocytes and alveolar macrophages, J. Immunol. 150 (7) (1993) 2885–2891.
[20] M. Qubaja, B. Marmey, A. Le Tourneau, S. Haiat, D. Cazals-Hatem, B. Fabiani, et al., The detection of cd14 and cd16 in paraffin-embedded bone marrow biopsies is useful for the diagnosis of chronic myelomonocytic leukemia, Virchows Arch. 454 (4) (2009) 411–419, http://dx.doi.org/10.1007/s00428-009-0726-x.
[21] S. Takeshita, K. Nakatani, H. Kawase, S. Seki, M. Yamamoto, I. Sekine, et al., The role of bacterial lipopolysaccharide-bound neutrophils in the pathogenesis of Kawasaki disease, J. Infect. Dis. 179 (2) (1999) 508–512, http://dx.doi.org/10.1086/314600.
[22] C. Krüger, C. Schütt, U. Obertacke, T. Joka, F.E. Müller, J. Knöller, et al., Serum cd14 levels in polytraumatized and severely burned patients, Clin. Exp. Immunol. 85 (2) (1991) 297–301.
[23] R. Beschorner, T.D. Nguyen, F. Gözalan, I. Pedal, R. Mattern, H.J. Schluesener, et al., Cd14 expression by activated parenchymal microglia/macrophages and infiltrating monocytes following human traumatic brain injury, Acta Neuropathol. 103 (6) (2002) 541–549, http://dx.doi.org/10.1007/s00401-001-0503-7.
[24] K.G.C. Smith, M.R. Clatworthy, Fcgammariib in autoimmunity and infection: evolutionary and therapeutic implications, Nat. Rev. Immunol. 10 (5) (2010) 328–343, http://dx.doi.org/10.1038/nri2762.
[25] J.V. Ravetch, S. Bolland, Igg fc receptors, Annu. Rev. Immunol. 19 (2001) 275–290, http://dx.doi.org/10.1146/annurev.immunol.19.1.275.
[26] C.L. Holness, D.L. Simmons, Molecular cloning of cd68, a human macrophage marker related to lysosomal glycoproteins, Blood 81 (6) (1993) 1607–1613.
[27] E. Kunisch, R. Fuhrmann, A. Roth, R. Winter, W. Lungershausen, R.W. Kinne, Macrophage specificity of three anti-cd68 monoclonal antibodies (kp1, ebm11, and pgm1) widely used for immunohistochemistry and flow cytometry, Ann. Rheum. Dis. 63 (7) (2004) 774–784, http://dx.doi.org/10.1136/ard.2003.013029.