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Influence of transfusion of lymphokine-activated T killer cells on inflammatory responses in dogs after laparotomy

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ABSTRACT. The influence of transfusion of lymphokine-activated T killer cells (T-LAK) on inflammatory responses was examined in dogs after laparotomy. Plasma C-reactive protein (CRP) level, cell numbers of peripheral blood lymphocytes (PBLs) and T lymphocyte subsets ($CD3^+$, $CD4^+$ and $CD8^+$) and mRNA expression levels of cytokines including interleukin (IL)-2, IL-12, IL-4, IL-10 and transforming growth factor (TGF)-β in peripheral blood mononuclear cells (PBMCs) were measured in dogs with (T-LAK group) or without (control group) a single T-LAK administration immediately after laparotomy. The plasma CRP level initially increased and then decreased to the normal range at 7 days after laparotomy in the T-LAK group, which was earlier than in the control group. The expression level of IL-10 mRNA showed a marked postoperative increase and was significantly higher than the preoperative level on day 7 ($P<0.05$), whereas the level in the control group showed no clear change after laparotomy. A significant increase in IL-2 mRNA expression level in the T-LAK group was observed on day 14, which was two weeks earlier than in the control group ($P<0.05$). These results suggest that T-LAK therapy in dogs after laparotomy leads to earlier resolution of postoperative inflammation by production of an anti-inflammatory cytokine (IL-10) in the early phase of the postoperative period and earlier restoration of cell-mediated immunity related to cytokine production by PBMCs.
KEY WORDS: anti-inflammatory cytokine, canine, cell-mediated immune cytokine, inflammation, lymphokine-activated T killer cell

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Surgery involves cutting of the body for repair or removal of diseased or damaged tissue. Inflammation occurs postoperatively, and the inflammatory response is necessary for tissue repair, but an excessive response can cause immunosuppression, followed by severe complications, such as sepsis and organ failure [13, 16]. Therefore, control of inflammatory responses is important for early tissue repair, prevention of severe complications and a favorable prognosis [19].

Transfusion therapy with lymphokine-activated T killer cells (T-LAK) is used as immunotherapy for cancer patients and for dogs with cancer [5, 7, 15]. T-LAK therapy induces restoration of cell-mediated immune responses and has been shown to prevent immunosuppression, which is one of the major postoperative complications associated with inflammation after surgery [9, 13, 16, 19].

Lymphocytes, especially T lymphocytes, influence inflammatory responses by producing anti-inflammatory cytokines, such as interleukin (IL)-4 [4], IL-10 [3, 11, 14] and transforming growth factor (TGF)-β [21], and influence cell-mediated immune responses by secreting cytokines, such as IL-2 and IL-12. A decrease of peripheral blood lymphocytes (PBLs) was observed in humans and dogs after surgery [17–19], and T-LAK therapy prevents postoperative reduction of PBLs in humans [19]. T-LAK therapy also influences cytokine production of PBLs [6]. Although these suggest that T-LAK therapy may prevent postoperative decrease of PBLs and affect postoperative production of anti-inflammatory cytokines and cell-mediated immune cytokines in PBLs in dogs after surgery, few studies have been done concerning changes of PBLs numbers and production of anti-inflammatory cytokines and cell-mediated immune cytokines in PBLs in dogs with T-LAK therapy after surgery. In this study, effects of T-LAK therapy after laparotomy on the plasma C-reactive protein (CRP) level; numbers of peripheral blood neutrophils (PBNs), PBLs and T lymphocyte subsets; and mRNA expression levels of IL-2, IL-12 p35, IL-12 p40, IL-4, IL-10 and TGF-β in peripheral blood mononuclear cells (PBMCs) were evaluated in dogs.

MATERIALS AND METHODS

Experimental animals: Ten healthy beagle dogs (males,

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1 year old) were used in this study. The dogs were divided into 2 groups of 5 dogs each. One group underwent laparotomy without postoperative T-LAK therapy (control group), and the other received laparotomy with postoperative T-LAK therapy (T-LAK group). The study was conducted according to the Guidelines of the Experimental Animal Committee of Obihiro University of Agriculture and Veterinary Medicine (Permit number 21–132).

Experimental procedure: The dogs were injected subcutaneously with atropine sulfate (0.025 mg/kg, Fuso Pharmaceutical Industries, Osaka, Japan) and meloxicam (0.2 mg/kg, Metacam, Boehringer Ingelheim Vetmedica Japan, Tokyo, Japan) before anesthesia. General anesthesia was induced by intravenous administration of thiamylal sodium (12.5 mg/kg, Isozol, Nichi-Iko Pharmaceutical, Toyama, Japan) following intravenous injection of midazolam (0.2 mg/kg, Dormicum, Astellas Pharma, Tokyo, Japan) and butorphanol (0.2 mg/kg, Vetorphale, Meiji Seika Pharma, Tokyo, Japan). An endotracheal tube was placed into the trachea to facilitate control of respiration. Anesthesia was maintained with 1.5–2.0% isoflurane (Mylan Seiyaku, Tokyo, Japan) and oxygen. The dogs were placed in a ventrodorsal recumbent position. An experimental midline laparotomy from the xiphoid to umbilicus was performed. After observation of organs in the abdominal cavity, abdominal and skin closure were carried out by routine procedures with synthetic absorbable (Biosyn, Covidien Japan, Tokyo, Japan) and nonabsorbable (Monosof, Covidien Japan) suture materials.

Peripheral blood samples were collected by using venipuncture before (day 0) and on days 1, 3, 7, 14 and 28 after surgery. Samples were treated with EDTA prior to a complete blood count test, and heparinized samples were used for isolation of plasma and PBMCs.

A complete blood count test was performed with an automatic cell counter (Celltac α, Nihon Kohden, Tokyo, Japan) and microscopic examination of blood smears stained using a rapid stain kit (Hemacolor, Merck, Darmstadt, Germany). Cell numbers of PBNs and PBLs were calculated from the manual white blood cell (WBC) differential and the total WBC number. Plasma CRP level was measured with a Laser CRP-2 (Arrows, Osaka, Japan) according to the manufacturer's instructions.

Isolation of PBMCs: Heparinized blood was diluted with the same volume of phosphate-buffered physiological saline (PBS), and 10 m*l* of the diluted blood was layered on 3 m*l* of Lymphocyte Separation Solution (specific gravity: 1.077; Nacalai Tesque, Kyoto, Japan). The layered blood was centrifuged at 2,000 rpm for 45 min at 20°C, and then, the buffy coat was collected and suspended in the same volume of PBS. The cell suspension was then centrifuged at 1,600 rpm for 6 min at 20°C. The supernatant was removed, and the cell pellet was suspended in an appropriate volume of PBS. The cells were used immediately in further assays as PBMCs. All centrifugations were performed with a KC-70 rotor (Kubota, Tokyo, Japan).

Flow cytometric analysis of PBMCs: T lymphocyte subsets in PBMCs were analyzed by flow cytometry (EPICS XL, Beckman Coulter, Miami, FL, U.S.A.). Fifty microliters of a PBMC suspension adjusted to 2×10^6 cells/m*l* with PBS was dispensed into each of 3 tubes. Ten microliters of FITC-labeled anti-canine CD3 antibody (AbD Serotec, Oxford, U.K.) was added to one tube. Ten microliters of FITC-labeled anti-canine CD4 antibody/R-PE-labeled anti-canine CD8 antibody (AbD Serotec) was added to the second tube. The third tube served as a non-antibody control. The three tubes were incubated for 30 min at 4°C, and then, the cell suspension in each tube was mixed with an appropriate volume of PBS. The tubes were then centrifuged at 1,600 rpm for 6 min at 20°C with a KC-70 rotor. The cell pellets were suspended in 500 μl of sheath solution (IsoFlow sheath fluid, Beckman Coulter) and were then used for flow cytometric analysis.

Relative quantification of cytokine mRNA expression in PBMCs: PBMCs adjusted to 4×10^6 cells were lysed with 750 μl of ISOGEN (Nippon Gene, Toyama, Japan) and left to stand for 5 min at 20°C. The lysate was applied to a QIA shredder column (Qiagen, Tokyo, Japan) and centrifuged at 12,000 rpm for 2 min at 4°C. The cell lysate was mixed with 200 μl of chloroform, shaken vigorously on a vortex mixer and then left to stand for 3 min at 20°C. The mixture was then centrifuged at 12,000 rpm for 15 min at 4°C, and the aqueous phase was mixed with 500 μl of 2-propanol and left to stand for 10 min at 20°C. The solution was centrifuged at 12,000 rpm for 10 min at 4°C. The precipitate was washed with 1 m*l* of 70% ethanol and centrifuged at 7,500 rpm for 5 min at 4°C, and the precipitate was dried after removing the supernatant. The precipitate was dissolved in 10 μl of diethylpyrocarbonate (DEPC)-treated water. The total RNA content was quantified by a spectrophotometer (Eppendorf, Hamburg, Germany). Centrifugations in this section were performed with a Model 3520 centrifuge (Kubota).

One microgram of the extracted total RNA was mixed with 0.5 μl of oligo (dT) 12–18 (Amersham Pharmacia Biotech, Swampscott, MA, U.S.A.), 4 μl of 5×buffer for ReverTra Ace, 2 μl of 10 mM PCR nucleotide mix (GE Healthcare, Little Chalfont, U.K.) and 1 μl of ReverTra Ace (Toyobo, Osaka, Japan), and then, the total volume was adjusted to 20 μl by adding DEPC-treated water. The mixed solution was reacted at 30°C for 10 min, at 42°C for 60 min and at 99°C for 5 min for cDNA synthesis.

Real-time PCR was performed for relative quantification of IL-2, IL-4, IL-10, IL-12 p35, IL-12 p40 and TGF-β1 mRNA expression in PBMCs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and relative expression levels of cytokine mRNA were estimated. To examine each expression level, the preoperative level was defined as 1. The primer pairs used in the study are shown in Table 1. Five microliters of the synthesized cDNA solution was mixed with 10 μl of water, PCR grade, 0.2 μl of the applicable Universal ProbeLibrary probe (Roche Diagnostics, Mannheim, Germany), 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reverse primer and 4.0 μl of LightCycler TaqMan Master (Roche Diagnostics). The solution was infused into a LightCycler Capillary (Roche Diagnostics) and centrifuged at 3,000 rpm for 30 sec at 20°C with an LC Carousel Centrifuge 2.0 (Roche Diagnostics).

Table 1. List of primer pairs

Primer		Sequence (5′-3′)	Length of amplicon
GAPDH	F	ATGATTCTACCCACGGCAAA	76 bp
	R	ATGGACTTCCCGTTGATGAC	
IFN-γ	F	CGGTGGGTCTCTTTTCGTAG	72 bp
	R	TGGCTCTGAATGATTGTTTTGT	
IL-2	F	CAGAGCAACAGATGGAGCAA	61 bp
	R	TTATTAACTCCATTCAAAAGCAACTG	
IL-4	F	CAGATATCTCAGAGGACTCTACAGGA	75 bp
	R	TTCTTGATTTCATTCATAGAACAGGT	
IL-10	F	TGGGAGAGAAGCTCAAGACC	66 bp
	R	TCTCACAGGGCAGAAATCG	
IL-12 p35	F	GGAAAGGCCTCTTTTATGACG	70 bp
	R	CCATCTGGTACATCTTCAAGTCC	
IL-12 p40	F	GCGTCTTCCCTCATGACC	68 bp
	R	GGGTGCCAGTCCAACTCTAC	
TGF-β1	F	TGGCTGTCCTTTGATGTCAC	71 bp
	R	CGAAAGCCCTCGACTTCC	

F: forward primer, R: reverse primer.

After centrifugation, real-time PCR was performed using a LightCycler 1.5 instrument (Roche Diagnostics). The reaction conditions were as follows: 10 min at 95°C, followed by 45 cycles of 10 sec at 95°C and 30 sec at 60°C, and a final 30 sec at 40°C.

T-LAK therapy: The bottom of a 250 m*l* flask for suspension cell culture (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) was coated with 10 m*l* of 5 μg/m*l* anti-canine CD3 antibody solution diluted with Dulbecco's PBS (D-PBS; Nacalai Tesque). The flask was allowed to stand at 4°C overnight. PBMCs isolated from 20 m*l* of peripheral blood were suspended in 20 m*l* of recombinant human (rh) IL-2-containing culture medium (LAM-1, Canine-Lab, Tokyo, Japan) with 2.5% fetal bovine serum (FBS; Biowest, Nuaille, France). The cell suspension was cultured in the flask coated with anti-CD3 antibody at 37°C under 5% CO_2. On days 3 and 6, 20 m*l* of culture medium containing rhIL-2 (LAM-2, Canine-Lab) was added. On day 7, the cell suspension was transferred to a culture bag with culture medium containing rhIL-2 (LAM-3, Canine-Lab) from the flask. On day 14, the cultured cells were harvested from the bag and washed twice with sterile physiological saline. Finally, the cells were suspended in 50 m*l* of sterile physiological saline and filtrated through a sterile 100-μm nylon mesh. Total cell counts and percentages of T lymphocyte subsets ($CD3^+$, $CD4^+$ and $CD8^+$) in the suspension were investigated as described above before administration to dogs. The expression level of interferon (IFN)-γ was also evaluated by real-time PCR. The suspension of T-LAK was injected intravenously into each dog in the T-LAK group immediately after laparotomy.

Statistical analysis: All results are shown as mean ± standard deviation. A comparison between T-LAK and PBMCs was performed with the paired *t* test. A comparison between the T-LAK group and control group was performed with two-way ANOVA and the Tukey-Kramer method. Statistical significance was set at $P<0.05$. All analyses were performed using Statcel 3, a Microsoft Excel plug-in (OMS Publishing, Saitama, Japan).

RESULTS

The total number of cells administered by T-LAK therapy was $6.4 \pm 1.6 \times 10^8$ cells. The percentages of the T lymphocyte subsets of T-LAK were 99 ± 0.4% for $CD3^+$, 30 ± 9.3% for $CD4^+$, 69 ± 9.2% for $CD8^+$ and 24 ± 6.8% for $CD4^+CD8^+$ cells. The IFN-γ mRNA expression level of T-LAK on day 14 was significantly higher than that in PBMCs before culture ($P<0.01$, Fig. 1).

The PBN cell numbers in the T-LAK and control groups increased immediately after laparotomy and reached a maximum on day 1 (Fig. 2A). The number in the T-LAK group returned to the preoperative number from day 3, whereas this return did not occur until day 7 in the control group. The plasma CRP levels increased immediately after laparotomy and reached a maximum on day 1 in both groups, and they were in the normal range from day 3 onwards in the T-LAK group and from day 7 onwards in the control group (Fig. 2B).

The IL-4 mRNA expression level tended to decrease after laparotomy, reached a minimum on day 7 and then significantly increased to above the preoperative level on day 14 in the T-LAK group ($P<0.05$, Fig. 3A). The IL-4 mRNA expression level in the control group showed no clear change after laparotomy. Although the IL-10 mRNA expression level in the control group showed no change after laparotomy, it increased markedly from day 3 after laparotomy and was significantly higher than all other expression levels on day 7 ($P<0.05$, Fig. 3B), and it then tended to decrease to the preoperative level in the T-LAK group. The TGF-β1 mRNA expression level decreased after laparotomy and reached a significantly lower level on day 7 than the preoperative level in the T-LAK group ($P<0.05$, Fig. 3C). The TGF-β1 mRNA expression level tended to increase on day 28 in both groups.

The PBL cell number increased after laparotomy, remained high on days 3 to 7 and returned to the preoperative

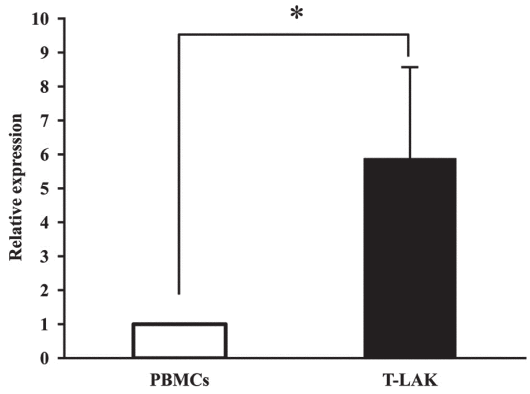

Fig. 1. IFN-γ mRNA expression levels in PBMCs and T-LAK. The black and white boxes represent T-LAK and PBMCs, respectively. *P<0.01 vs PBMCs.

value on day 28 in the T-LAK group (Fig. 4A). The $CD3^+$, $CD4^+$ and $CD8^+$ cell numbers in the T-LAK group showed similar changes (Fig. 4B–4D). The PBL cell number in the control group decreased after laparotomy, showed no change from days 1 to 7 and then returned to the preoperative value on day 28. The $CD3^+$, $CD4^+$ and $CD8^+$ cell numbers behaved similarly. The numbers of PBLs and all T lymphocyte subsets in the T-LAK group were significantly higher than those in the control group by two-way ANOVA (P<0.05).

The expression level of IL-2 mRNA in both groups tended to decrease after laparotomy and reached a minimum on day 7 (Fig. 5A). The IL-2 mRNA expression level was significantly higher than the preoperative level on days 14 and 28 in the T-LAK group and on day 28 in the control group (all P<0.05). The IL-12 p35 mRNA expression level tended to decrease after laparotomy and reached a minimum on day 7 in the T-LAK group and on day 3 in the control group (Fig. 5B). The IL-12 p35 mRNA expression level tended to increase on days 14 and 28 in the T-LAK group and on day 28 in the control group. The IL-12 p40 mRNA expression level tended to decrease after laparotomy, reached a minimum on day 7 and then increased on days 14 and 28 (Fig. 5C). The IL-12 p40 mRNA expression level in the control group did not change markedly from day 0 to day 28.

DISCUSSION

It is popular to produce T-LAK using autologous PBLs with solid phase anti-CD3 antibody and IL-2 in dogs [5, 7] and humans [15]. In the present study, the number of cultured lymphocytes increased approximately 46-fold, and the main subset of cultured lymphocytes comprised $CD3^+$ cells. In addition, the IFN-γ mRNA expression level of cultured lymphocytes, which is one of the indicator cytokines of T-LAK [5], was significantly higher than that of PBMCs (Fig. 1). Therefore, the cultured lymphocytes in this study can be regarded as T-LAK.

The number of PBNs and plasma CRP level are considered markers for the presence and severity of inflammation

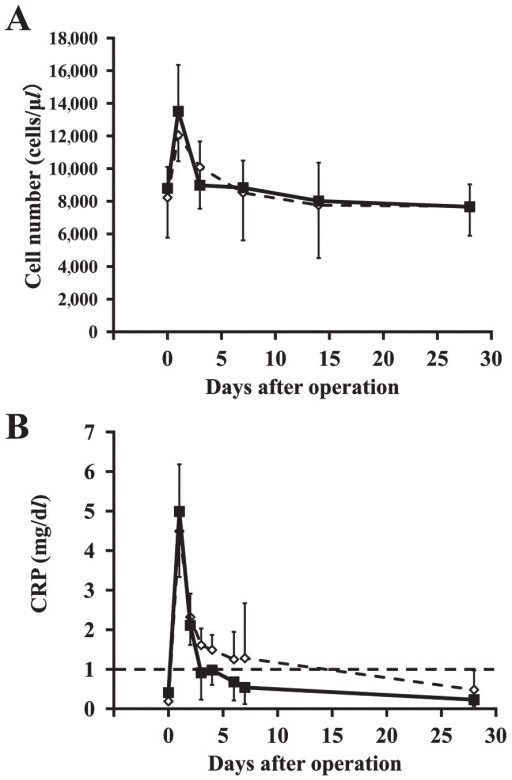

Fig. 2. Effects of T-LAK therapy on the number of PBNs and plasma CRP level. (A) Number of PBNs, (B) plasma CRP levels. The black squares and solid line represent the T-LAK group, and the white diamonds and dashed line represent the control group. The normal range for the plasma CRP level (<1 mg/d*l*) is shown by a dashed line.

[12, 20]. The number of PBNs and the plasma CRP level in the T-LAK group returned to the normal range earlier than those in the control group (Fig. 2). These results suggested that postoperative T-LAK therapy might provide earlier resolution of inflammation in dogs after laparotomy.

Resolution of inflammation requires anti-inflammatory cytokines, such as IL-4 [4], IL-10 [3, 11, 14] and TGF-β [21], and lymphocytes, especially T lymphocytes, control inflammatory responses by producing these cytokines. Of these anti-inflammatory cytokines, IL-10 is the most important anti-inflammatory cytokine, and an initial increase of IL-10 prevents systemic inflammation and severe postoperative complications [9, 10]. Although the IL-10 mRNA expression level in the control group showed no clear changes after laparotomy, a remarkable increase in the IL-10 mRNA expression level was observed in the early period after laparotomy, and this increase accompanied a decrease in the plasma CRP level to the normal range in the T-LAK group (Figs. 2B and 3B). This result indicated that the postoperative T-LAK therapy induced production of IL-10 in PBMCs, which was closely related to resolution of the early phase of inflammation in the dogs after laparotomy. It is possible that the inflammatory responses in the control group might be

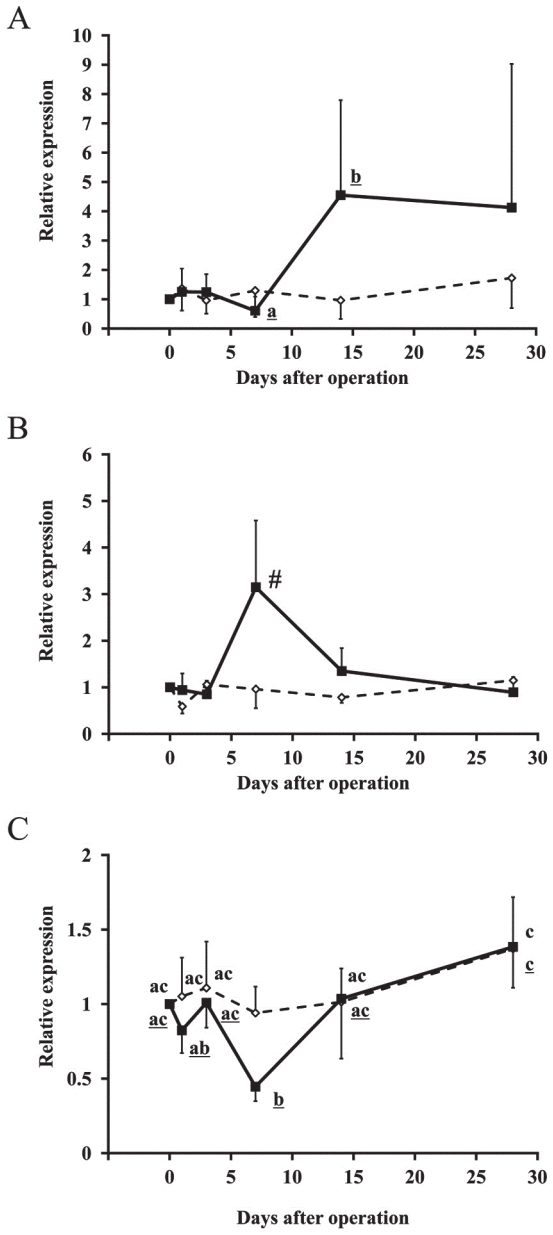

Fig. 3. Effects of T-LAK therapy on mRNA expression of anti-inflammatory cytokines in PBMCs. (A) IL-4, (B) IL-10, (C) TGF-β1. The black squares and solid line represent the T-LAK group, and the white diamonds and dashed line represent the control group. Different lowercase letters indicate significant differences (P<0.05). Lowercase letters with and without underlining indicate the levels in the T-LAK and control groups, respectively. #P<0.01 vs all other.

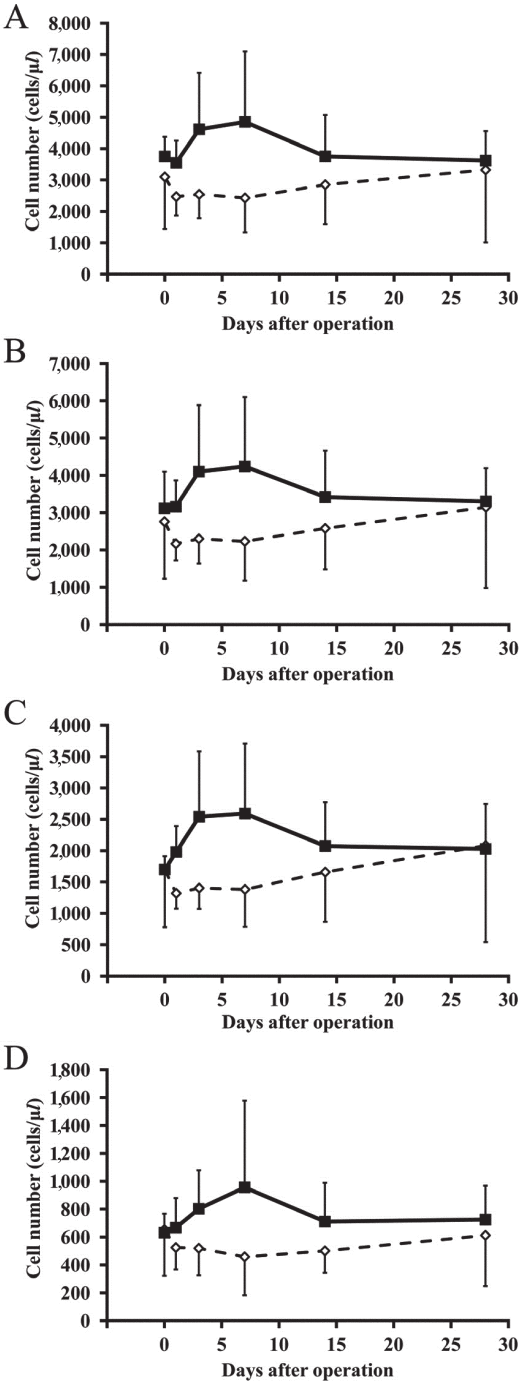

Fig. 4. Effects of T-LAK therapy on numbers of PBLs and T lymphocyte subsets. (A) PBLs, (B) $CD3^+$ cells, (C) $CD4^+$ cells, (D) $CD8^+$ cells. The black squares and solid line represent the T-LAK group, and the white diamonds and dashed line represent the control group. The numbers of PBLs and all T lymphocyte subsets in the T-LAK group were significantly higher than those in the control group by two-way ANOVA (P<0.05).

controlled by local anti-inflammatory responses, whereas the responses in the T-LAK group might be resolved by systemic anti-inflammatory responses. There are some studies that have reported that IL-10 is induced by the IFN-γ production of T lymphocytes for prevention of excessive inflammation [2, 8], and the IFN-γ-producing T-LAK are thought to be possibly associated with the induction of IL-10 production. On the other hand, the mRNA expression levels of IL-4 and

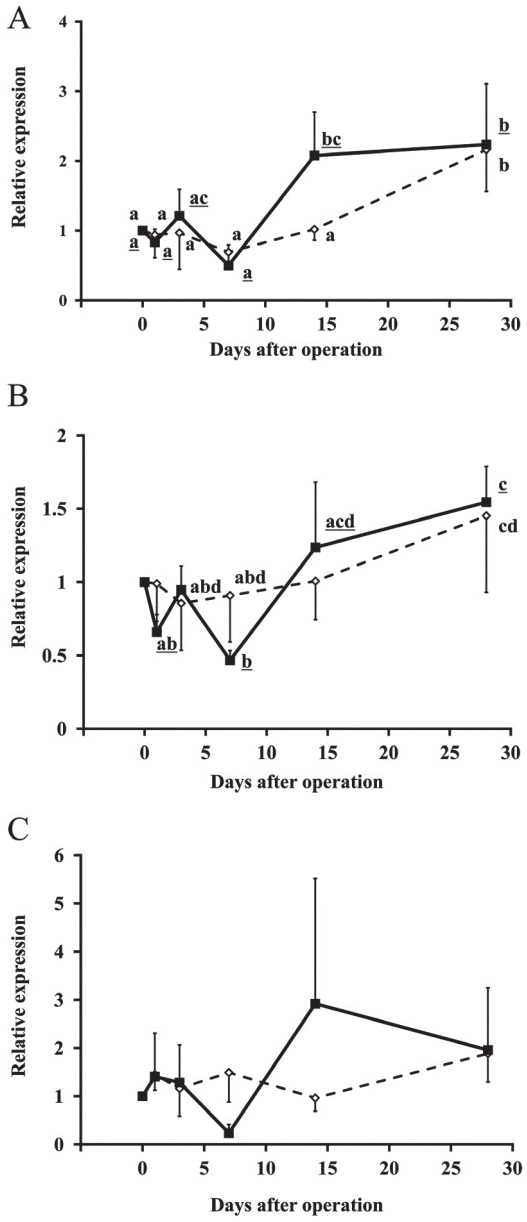

Fig. 5. Effects of T-LAK therapy on mRNA expression of cell-mediated immune cytokines in PBMCs. (A) IL-2, (B) IL-12 p35, (C) IL-12 p40. The black squares and solid line represent the T-LAK group, and the white diamonds and dashed line represent the control group. Different lowercase letters indicate significant differences ($P<0.05$). Lowercase letters with and without underlining indicate the levels in the T-LAK and control groups, respectively.

TGF-β increased after CRP decreased to the normal range in the T-LAK group (Figs. 2B, 3A and 3C). These results indicated that IL-4 and TGF-β production was unrelated to control of inflammatory responses in the early phase after laparotomy. IL-4 [9] and TGF-β [1] promote wound healing after injury, and increases in IL-4 and TGF-β production caused by T-LAK therapy are likely to be part of the wound healing response rather than resolution of the early phase of postoperative inflammation.

Lymphocytes, especially T lymphocytes, are important in control of inflammation [13, 16]. Yamada *et al.* [18] showed that the numbers of PBLs and $CD3^+$ cells decrease immediately in dogs after laparotomy and recover to the preoperative values by the 4th week after that. The postoperative decrease of the numbers of PBLs is thought to be associated with severe complications, such as sepsis and organ failure [17, 19]. Although the numbers of PBLs and $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes decreased in the control group immediately after laparotomy, the T-LAK group showed no decrease in the numbers of PBLs and the three lymphocyte subsets after laparotomy (Fig. 4). It is suggested that postoperative T-LAK therapy could prevent the loss of PBLs, $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes in dogs after laparotomy. The PBLs in the T-LAK group may stimulate production of IL-10 by the T lymphocyte subsets in the early period after laparotomy.

T-LAK therapy is considered to activate cell-mediated immune response [6, 19]. IL-2 and IL-12 are important cytokines for cell-mediated immune responses, and surgical trauma has been known to suppress production of these cytokines by T lymphocytes in association with postoperative inflammation [9, 16]. The expression level of IL-2 mRNA in the T-LAK group increased to a significantly higher level, and this occurred earlier than in the control group (Fig. 5A). The expression levels of IL-12 p35 and IL-12 p40 mRNA in the T-LAK group also tended to increase similarly to IL-2 mRNA (Fig. 5B and 5C). Interestingly, increase of the mRNA expression levels of cell-mediated immune cytokines was observed after the resolution of postoperative inflammation in the T-LAK group. It is suggested that T-LAK therapy might induce earlier restoration of cell-mediated immune responses by resolution of postoperative inflammation in dogs after laparotomy.

In conclusion, T-LAK therapy in dogs after laparotomy could enable earlier resolution of inflammation, could induce IL-10 production by PBMCs in the early phase of postoperative inflammation and could enable earlier restoration of production of cell-mediated immune cytokines (IL-2 and IL-12) in PBMCs. It is suggested that resolution of postoperative inflammation brings about the earlier restoration of cell-mediated immune responses in dogs with T-LAK therapy after laparotomy. Although further studies are required to evaluate the association between T-LAK therapy and postoperative inflammatory responses in dogs, T-LAK therapy may be effective for resolution of inflammation and restoration of the cell-mediated immune responses in dogs after surgery.

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