Comparison of stimulating effect on subpopulations of lymphocytes in human peripheral blood by methionine enkephalin with IL-2 and IFN- γ

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Abbreviations: MENK, methionine enkephalin; FCM, flow cytometry; IL-2, Interleukin-2; IFN- γ , Interferon- γ ; DC, dendritic cell; NK, natural killer; Treg, CD4+CD25+ regulatory T cells; PBML, peripheral blood mononuclear cell; RT-PCR, reverse transcriptase polymerase chain reaction

The aim of this study was to investigate the effects of mechanisms of methionine enkephalin (MENK) on lymphocytes in human peripheral blood. We detected CD4+T cells, CD8+T cells, CD4+CD25+ regulatory T cells (Treg), dendritic cells (DCs), natural killer cells (NK), NKT cells and $\gamma\delta$ T cells before and after treatment with 10^{-12} M MENK, in cell culture by FCM and RT-PCR. Our findings show that MENK stimulating expansion of lymphocyte subpopulations by inhibiting CD4+CD25+ regulatory T cells (Treg), which is unique discovery of our study. We may use MENK as a drug to treat cancer patients, whose immune systems are damaged by chemotherapy or radiotherapy.

Introduction

MENK, a penta-peptide is generated in adrenal gland and derived from proenkephalin. The endogenous neuropeptide, is suggested to be involved in the regulatory net between the immune and neuroendocrine systems, with modulation of various functions of cells related to both innate and adaptive immune systems in a naltrexone reversible manner.¹ Since the pioneer discovery by Wybran, revealing that there were enkephalin receptors on human T cells and subsequent findings that the enkephalins indeed activated T cells and NK cells as well as LAK cells and TIL cells by Plotnikoff and many others,² the endogenous opioid ligand interacting with more than one type of delta receptors have been detected to affect various immune cells, such as T cell,³ NK cells,^{4,7} macrophage^{8,9} and DCs¹⁰⁻¹³ at a moderate range of concentrations. MENK may work as a tonic activation agent that plays a role in immune modulation and cell proliferation. We recently demonstrated that MENK could exert positive modulation to the pathway of the DC-CD4+T cells in vitro and in vivo through increasing expression of delta receptors on the surface of the CD4+T cells and binding to delta receptors, which highlight a need to overcome the present bottleneck we are encountering in clinical application of IL-2 and IFN- γ for T cell mediated immune responses.¹⁴

Both IL-2 and IFN- γ are T cell activators via either autocrine or paracrine signaling, and play important roles in promoting T cell mediated immune responses in quite some of cancer patients. However, the applications of either IL-2 or IFN- γ in cancer therapy and other immune handicapped cases are very limited because of their side effects, limited efficacy or other unclear mechanisms.

Due to the great importance of restoring immune system in maintaining immune system homeostasis in the body and the role of balanced immunity in fighting cancers and other human-threatening diseases, like AIDS, especial role of the balanced immunity in cancer patient, whose immune system is damaged severely with chemotherapy and since the precise mechanisms of MENK's effect on balanced immunity in lymphocyte subpopulations remain unclear.¹⁵ We endeavored to conduct following exploration to try to reveal mechanisms, by which MENK is regulating cells of immune system, so as to support clinical application of MENK as a new drug fighting cancers in the future.

Results

The expansion of the CD4+CD25+Foxp3+T cells were inhibited after treatment with 10^{-12} M MENK, and this was confirmed by FCM. The positive percentage for the Treg yielded $6.21 \pm 0.75\%$

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in the MENK group, ($p < 0.05$) vs. $9.64 \pm 0.86\%$ in the RPMI 1640 group, ($p < 0.01$) vs. $10.38 \pm 0.52\%$ in the IL-2 group and ($p < 0.05$) vs. $9.57 \pm 0.54\%$ in the IFN- γ group. Neither IL-2 nor IFN- γ showed significant inhibition to Treg cells (Fig. 1A). Concurrently the expression of the CD4, CD25, Foxp3 molecules were reconfirmed at mRNA level by RT-PCR. These results indicated that MENK did downregulate the expression of these marker molecules on the Treg cells as shown in (Fig. 1B).

The data shown in (Fig. 2A) indicated the proportion of CD8+CD28+ cells after treatment with 10^{-12} M MENK for 48 h. RT-PCR analysis showed a significant increase of each molecule at mRNA level in the cells cultured in the presence of 10^{-12} M MENK. Moreover, the mRNA level was much higher as compared with the levels in either IL-2 group or IFN- γ group as reflected in (Fig. 2B).

The positive percentage for the NK cells yielded $7.47 \pm 0.35\%$ in the MENK group ($p < 0.01$) vs. $4.98 \pm 0.59\%$ in the RPMI 1640 group, ($p > 0.05$) vs. $6.84 \pm 0.54\%$ in the IL-2 group and with $p < 0.05$ vs. $5.94 \pm 0.59\%$ in the IFN- γ group, as shown in (Fig. 3A). Also the positive percentage for the NKT cells yielded $7.24 \pm 0.65\%$ in the MENK group, ($p < 0.05$) vs. $5.74 \pm 0.19\%$ in the RPMI 1640 group, ($p > 0.05$) vs. $6.59 \pm 0.51\%$ in the IL-2 group, ($p > 0.05$) vs. $6.45 \pm 0.28\%$ in the IFN- γ group (Fig. 3B).

The result shown in Figure 3C reflected the mRNA levels of CD3, CD16, CD56, essentially consistent to the data by FCM.

The positive percentage for the $\gamma\delta$ T cells yielded $9.08 \pm 0.43\%$ in the MENK group, ($p < 0.01$) vs. $6.21 \pm 0.7\%$ in the RPMI 1640 group, ($p < 0.05$) vs. $7.43 \pm 0.4\%$ in the IL-2 group and ($p < 0.05$) vs. $7.08 \pm 0.38\%$ in the IFN- γ group, as shown in (Fig. 4A). As shown in (Fig. 4B), RT-PCR analysis showed a significant increase of the $\gamma\delta$ T molecule at mRNA level in cells cultured in the presence of 10^{-12} M MENK.

The positive percentage for the DCs, CD1a+ yielded $1.46 \pm 0.09\%$ in the MENK group, ($p < 0.01$) vs. $0.77 \pm 0.09\%$ in the RPMI 1640 group, ($p < 0.05$) vs. $0.65 \pm 0.2\%$ in the IL-2 group and ($p < 0.05$) vs. $0.91 \pm 0.09\%$ in the IFN- γ group, as reflected in (Fig. 5A). The mRNA level of CD1a molecule was much higher compared with this in the IL-2 group and that in the IFN- γ group (Fig. 5B). The result indicated that MENK did enhance the expression of CD1a molecule.

Discussion

As an important transmitter connecting the endocrine and immune systems, MENK plays an important role in both endocrine and immune regulation. Moreover, the influence of this

Figure 1. The percentage of Treg in the lymphocytes after treatment with 10^{-12} M MENK for 48h by FCM (A) and reconfirmation of CD4, CD25, Foxp3 by RT-PCR at mRNA level (B). After treated with MENK, IL-2 and IFN- γ respectively in vitro, MENK inhibited Treg expansion in some direct ways as visualized by FCM profile. The number of Treg cells decreased substantially. Lane M: DNA marker; Lane 1: β -actin; Lane 2: RPMI-1640 group; Lane 3: β -actin; Lane 4: IFN- γ group; Lane 5: β -actin; Lane 6: IL-2 group; Lane 7: β -actin; Lane 8: MENK group. * $p < 0.05$ vs. in the RPMI1640 group; ** $p < 0.01$ vs. in the RPMI1640 group; # $p < 0.05$ vs. in the MENK group; ## $p < 0.05$ vs. in the MENK group. Results represent the mean $\pm$ SD of six samples.

Figure 2. The percentage of CD8+CD28+ cells in the lymphocytes after treatment with 10^{-12} M MENK for 48h (A) by FCM and reconfirmation of CD8, CD28 molecules by RT-PCR (B). Lane M: DNA marker; Lane 1: β -actin; Lane 2: RPMI-1640 group; Lane 3: β -actin; Lane 4: IFN- γ group; Lane 5: β -actin; Lane 6: IL-2 group; Lane 7: β -actin; Lane 8: MENK group. * $p < 0.05$ vs. in the RPMI1640 group; ** $p < 0.01$ vs. in the RPMI1640 group; # $p < 0.05$ vs. in the MENK group; ## $p < 0.05$ vs. in the MENK group. Results represent the mean $\pm$ SD of six samples.

molecule is shared between the neuroendocrine and immune systems.¹ In fact, MENK's function of upregulating immune system at suitable doses had been reported previously.¹⁶⁻¹⁸ The data of their findings proved that MENK could upregulate the functions of both innate and adaptive immune cells, including: (A) activation of neutrophils,^{19,20} NK cells,^{4,7,16,17} macrophages^{8,9,21} and T cells;^{3,22,23} (B) upregulation of the production of cytokines such as IL-2, IFN- γ , TNF- α ^{17,24} and IL-6 and (C) enhance of release of hydrogen peroxide and nitric oxide by macrophage.⁹

Our previous study had already shown that MENK could markedly stimulate CD4+T cell expansion and induce maturation of DCs with higher level of IL-12 production, which would intensify DC-CD4+T cell pathway, resulting in increased Th1 responses.¹⁴ Also we found, by our another study, MENK restored immune cells of peripheral blood in 21 cancer patients whose immune systems had been severely damaged by chemotherapy.²⁵

The present results obtained above, show that MENK's stimulating effect on the expansion of lymphocyte subpopulations in human peripheral blood was present and functional. In fact, what we found from our data are the following findings: (1) MENK at used concentration, could markedly stimulate the expansion of CD4+T cells (Fig. 1), CD8+T cells (Fig. 2), NK, NKT cells (Fig. 3), $\gamma\delta$ T cells (Fig. 4) and DCs (Fig. 5), which will directly result in increased immunity to remove antigens out of body and keep the body with perfect anti-defense, homeostasis and

surveillance, (2) MENK at used concentration could markedly inhibit the expansion of Treg, which reveals a new mechanism by which MENK exerts positive regulation to the cells of immune system and therefore this is unique discovery of this study with great potential in cancer therapy, (3) the marked expansion of $\gamma\delta$ T cells is with great importance in cancer therapy according to recently increasing number of data elucidating this point, and (4) MENK is more active than either IL-2 or IFN- γ in stimulating expansion of lymphocyte subpopulations by inhibiting Treg cells, which is a new mechanism to activate cellular immune system. MENK can act on human lymphocytes, promoting lymphocyte growth, improve and enhance human immunity. Also present data will help us with better understanding the mechanism, via which MENK is working in restoring human immune system, especially in concrete subpopulations of lymphocytes, such as DCs, NK, NKT cells and $\gamma\delta$ T cells.

It may be further noted that both IL-2 and IFN- γ do upregulate the expansion of CD8+T cells, DCs, NK, NKT cells and $\gamma\delta$ T cells. However, the impacts on these cells are limited, especially on $\gamma\delta$ T cells and they show no great inhibition to the Treg cells, which might be key obstacle for them to play more role and less efficacy observed in a large number of cases clinically.

As we know, the increased DCs not only augment the increase of CD4+T cells, but also increase the stability of the pathway between DCs and CD4+T cells, which, in turn, will result in more

Figure 3. The percentage of NK (A), NKT (B) cells in the lymphocytes after treatment with 10^{-12} M MENK for 48h by FCM and reconfirmation of CD3, CD16 and CD56 molecules by RT-PCR (C). The NK cells positive percentage generated in the MENK group was higher than these in the IFN- γ group ($p < 0.05$), however there was no significant difference with those in the IL-2 group ($p > 0.05$). Evidently either IL-2 or IFN- γ didn't stimulate marked expansion of NKT cells. Lane M: DNA marker; Lane 1: β -actin; Lane 2: MENK group; Lane 3: β -actin; Lane 4: IL-2 group; Lane 5: β -actin; Lane 6: IFN- γ group; Lane 7: β -actin; Lane 8: RPMI-1640 group. * $p < 0.05$ vs. in the RPMI1640 group; ** $p < 0.01$ vs. in the RPMI1640 group; # $p < 0.05$ vs. in the MENK group and ## $p < 0.05$ vs. in the MENK group. Results represent the mean $\pm$ SD of six samples.

secretion of cytokines like IL-2 and IFN- γ by the CD4 $^{+}$ T cells, which will trigger a chain of immune responses. Furthermore, it should also be noted that DCs have emerged as the most potent and professional antigen presenting cells that possess the ability to stimulate naive T cells and initiate T cell responses, acting as messengers between the innate and adaptive immunities. They can thus potentially be used in therapeutic vaccines in cancer immunotherapy and for other threatening diseases.²⁶⁻³⁰

In contrast, Treg cells are known for their suppressive capacity on various immune reactions and are a specialized subpopulation of T cells that act to suppress activation of the immune

system and thereby maintain immune system homeostasis and tolerance to self-antigens.³¹⁻³³ The identification of Treg cells represents a milestone in the field of immunology and provides an explanation for T-cell mediated immunosuppression. Although Treg cells were originally identified for their ability to prevent organ-specific autoimmune disease in mice, emerging evidence suggests that Treg cells play a pivotal role in tumor immunity and contribute to tumor growth and progression, thereby having an important impact on the outcome of cancer patients.³⁴⁻³⁸ Finally, despite unique discovery for MENK in inhibiting Treg cells there are still more mechanisms for us to investigate further to gain

Figure 4. The percentage of $\gamma\delta$ T cells in the lymphocytes after treatment with 10^{-12} M MENK for 48h (A) by FCM and reconfirmation by RT-PCR of the expression of $\gamma\delta$ T cells at mRNA level (B). A higher percentage of $\gamma\delta$ T cells as visualized by FCM profile indicated that the cell number increased substantially. Lane M: DNA marker; Lane 1: RPMI-1640 group; Lane 2: β -actin; Lane 3: IFN- γ group; Lane 4: β -actin; Lane 5: IL-2 group; Lane 6: β -actin; Lane 7: MENK group; Lane 8: β -actin. * $p < 0.05$ vs. in the RPMI1640 group; ** $p < 0.01$ vs. in the RPMI1640 group; # $p < 0.05$ vs. in the MENK group and ## $p < 0.05$ vs. in the MENK group. Results represent the mean $\pm$ SD of six samples.

thorough understanding on MENK, such as concrete impact on signals to inhibit Treg cells (studying in process).

The immune system is a very complicated and diverse entity, controlled by endocrine system via signaling molecules that act as activating agents, and thus, formulating a coordinated interaction between the immune and endocrine systems. In addition, there are internal interactions among the immune cells through a variety of cytokines that regulate or even police each other as part of a dynamic immune system.

MENK works as an immune modulator in restoring human immune system and we may use MENK as a drug to treat cancer patients, whose immune systems are damaged by chemotherapy or radiotherapy. Also we may consider MENK as a chemotherapy additive, which will sustain immune system of a cancer patient during the process of chemotherapy to get maximized treatment with minimized side effects at same time. In this way the life of a cancer patient could be prolonged markedly or even saved.

This study can therefore contribute to the understanding in depth, of MENK's positive modulating effects on the immune system. Our results also provide a meaningful mechanism of action for MENK and highlight the clinical significance of MENK in cancer immunotherapy, especially for terminal cancer patients and patients whose immune systems are severely damaged by chemotherapy.³⁹⁻⁴¹ According to our previous study in cancer therapy with MENK, the application of MENK should

include: (1) the application of MENK, before chemotherapy or with chemotherapy would sustain the immune system, show great synergistic with drugs, and minimize side effects caused by drugs during process of chemotherapy, (2) the application of MENK, post chemotherapy would restore immune system much fast, and minimize side effects caused by drugs during process of chemotherapy. Likewise, we may consider MENK as a possible adjuvant to be used in vaccine preparations against life-threatening diseases like AIDS.

Materials and Methods

Reagents. MENK was provided by Penta Biotech. Inc. US ($\geq 97\%$ purity). In our previous pre-experiments we tested a range of concentrations of MENK from 10^{-1} to 10^{-15} M on the proliferation of human lymphocyte in vitro and proved the optimal concentration of 10^{-12} M. So based on our previous studies, the optimal concentration of 10^{-12} M of MENK was used in this study.¹⁴ For MENK solution the powdered MENK was dissolved in RPMI1640 to make a 10^{-1} M solution and would be diluted to designated concentration for use. Recombinant human IFN- γ (Catalog number: 20070809), and recombinant human IL-2 (Catalog number: 20070806) were purchased from PEPROTECH Inc. The mAbs used in this study: FITC anti-human CD8a, PE anti-human CD28, PerCP anti-human

Figure 5. The percentage of DCs after treatment with 10^{-12} M MENK for 48h by FCM (A) and reconfirmation by RT-PCR of the expression of CD1a molecule at mRNA level (B). Lane M: DNA marker; Lane 1: β-actin; Lane 2: MENK group; Lane 3: β-actin; Lane 4: IL-2 group; Lane 5: β-actin; Lane 6: IFN-γ group; Lane 7: β-actin; Lane 8: RPMI-1640 group. * $p < 0.05$ vs. in the RPMI1640 group; ** $p < 0.01$ vs. in the RPMI1640 group; # $p < 0.05$ vs. in the MENK group and ## $p < 0.05$ vs. in the MENK group. Results represent the mean $\pm$ SD of six samples.

CD3, FITC anti-human CD16, PE anti-human CD56, FITC anti-human TCR γ/δ , FITC anti-human CD1a and Human Treg Flow™ Kit were all purchased from Biolegend. Ficoll-Paque solution was a product of sigma-Wisconsin. RT-PCR Kits were purchased from Takra. RT-PCR primer was prepared by Nanking Kinsit Gene Tech. Co. China. Trizol was a product of Invitrogen. Other chemicals frequently used in our laboratory were all products from sigma-Aldrich or BD PharMingen.

Subject. All reported data are derived from experiments using PBMC of the 6 healthy individuals.

Lymphocytes isolation and culture in vitro. Peripheral blood mononuclear cells PBMCs were isolated from heparinized human peripheral blood of six healthy volunteers aged from 18 y to 35 y (the mean age was 25.8 ± 5.6 y), who all signed informed consent, by Ficoll density gradient centrifugation. Freshly isolated PBMCs were washed twice with phosphate-buffered saline (PBS) and the cell vitality was tested by trypan blue dye exclusion assay. The cell concentration was adjusted to 1×10^6 /ml and the cells were grown in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1.2% sodium bicarbonate. All media contained antibiotics (100 units/ml penicillin, 100 μ g/ml streptomycin, 100 μ g/ml kanamycin). Unless otherwise indicated, all cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Analysis by flow cytometry (FCM). Lymphocyte subpopulations were measured by their immunophenotypes. We recorded the major lymphocyte subpopulations with CD8+CD28+ T cells

and Treg (CD4+CD25+Foxp3+), NK cells (CD16+CD56+), NKT cells (CD3+CD56+), $\gamma\delta$ T cells ($\gamma\delta$ TCR+), DCs (CD1a+). The lymphocytes grown as previously described, were then performed to the following groups; MENK 10^{-12} M, IL-2 (200 U/ml), IFN- γ (400 U/ml) and RPMI1640 (as control). Diversification of the lymphocyte subpopulations after 48 h were checked with FACS Calibur (Becton Dickinson) as well as confirmed by the RT-PCR analysis at mRNA level for each molecule marker. Typically 10,000 events were acquired in the gating region. Each subpopulation was expressed as percentage of lymphocytes and the data were then analyzed using WinMDI 2.9 software (Joseph Trotter, BD Biosciences).

Confirmation of each molecule by RT-PCR at mRNA level. The analysis of each molecule in a final volume of 50 μ l sample was performed by Takra two-step RT-PCR Kit per manufacturer's instructions. The RT-PCR reaction was performed as shown in Table 1.

Meanwhile, the total cellular RNA was extracted from the lymphocytes with TRIZOL, precipitated and resuspended in 50 μ l diethylpyrocarbonate treated-dH₂O, and the concentration was determined by optic density lecture at 260 nm using a GeneQuantPro spectrophotometer. The integrity of the RNA was tested by applying 1 μ l in 2% agarose gel containing ethidium bromide, and subsequently was visualized under UV transilluminator.

Statistical analysis. Statistical analysis was performed using the statistical program SPSS (Statistical Package for Social

Table 1. RT-PCR reaction of each molecule at mRNA level

Primer	Denaturation	Annealing	Extension
β -actin	94°C 30s	56°C 50s	72°C 1min
CD4	94°C 30s	56°C 50s	72°C 50s
CD25	94°C 30s	55°C 50s	72°C 50s
Foxp3	94°C 30s	56°C 50s	72°C 50s
CD3	94°C 30s	56°C 50s	72°C 1min
CD16	94°C 30s	55°C 50s	72°C 1min
CD56	94°C 30s	56°C 55s	72°C 1min
CD8	94°C 30s	56°C 50s	72°C 50s
CD28	94°C 30s	55°C 50s	72°C 50s
CD1a	94°C 30s	56°C 50s	72°C 1min
γ δ T	94°C 30s	56°C 50s	72°C 1min

The primer sequences are as follow:

Primer	Sequences	Size
β -actin	Sense 5' AGCCATGTACGTTGCTATCCAGGC 3'	500BP
	Antisense 5' TGTTGTCGTACAGGTCTTTGCGG 3'	
CD4	Sense 5' ACTCTGACACCCACCTGCTTCA 3'	246BP
	Antisense 5' TGCTGGAGGCCTTCTGGAAGCT 3'	
CD25	Sense 5' AAAAGAGGCTGACGGCAACTGGG 3'	213BP
	Antisense 5' TCCTTCCTTGTAGGCCATGGCT 3'	
Foxp3	Sense 5' TCATATCCTTTGCCCAAGGCTGC 3'	502BP
	Antisense 5' TGTGCGTGTGCATATGCGTGA 3'	
CD3	Sense 5' ACTGGACCTGGGAAAACGCATCC 3'	213BP
	Antisense 5' TGTCCAGCAAAGCAGAAGACTCCCA 3'	
CD16	Sense 5' AGTACAGGTGCCAGACAAACCTCTC 3'	282BP
	Antisense 5' TTTTACTCCCAACAAGCCCCCTGCA 3'	
CD56	Sense 5' CCGAGAGCCCACCATGAGCT 3'	333BP
	Antisense 5' TCGCTGCTGATGTTCCGGGT 3'	
CD8	Sense 5' ACAGAAAGGCTGATTGAGGCACACC 3'	180BP
	Antisense 5' GCCATGAGGACAGATGAGGGAACA 3'	
CD28	Sense 5' ACGGGGTTCAACTGTGATGGGA 3'	200BP
	Antisense 5' CCGGGAAATAGGGGACTTGGACA 3'	
CD1a	Sense 5' AGTTTGTACTGTCGACAGGGCA 3'	300BP
	Antisense 5' CCTTCCCCTAATTCAACCGCTATGG 3'	
γ δ T	Sense 5' TGACCTCGCAACAGAAAACCCAGA 3'	244BP
	Antisense 5' AGGGTTGCACACTTCTCCCCA 3'	

References

- Bhargava HN. Opioid peptides, receptors, and immune function. *NIDA Res Monogr* 1990; 96:220-33; PMID:2172824.
- Plotnikoff NP. Methionine enkephalin: a new cytokine-human studies. *J Clin Immunol Immunopathol* 1997; 82:93-101; <http://dx.doi.org/10.1006/clin.1996.4287>.
- Ohmori H, Fujii K, Sasahira T, Luo Y, Isobe M, Tatsumoto N, et al. Methionine-enkephalin secreted by human colorectal cancer cells suppresses T lymphocytes. *Cancer Sci* 2009; 100:497-502; PMID:19141128; <http://dx.doi.org/10.1111/j.1349-7006.2008.01073.x>.
- Hsueh CM, Chen SF, Huang HJ, Ghanta VK, Hiramoto RN. Activation of mu-opioid receptors are required for the conditioned enhancement of NK cell activity. *Brain Res* 1996; 737:263-8; PMID:8930374; [http://dx.doi.org/10.1016/0006-8993\(96\)00740-8](http://dx.doi.org/10.1016/0006-8993(96)00740-8).
- Burger RA, Warren RP, Huffman JH, Sidwell RW. Effect of methionine enkephalin on natural killer cell and cytotoxic T lymphocyte activity in mice infected with influenza A virus. *Immunopharmacol Immunotoxicol* 1995; 17:323-34; PMID:7650294; <http://dx.doi.org/10.3109/08923979509019754>.
- Kowalski J, Belowski D, Wielgus J. Bidirectional modulation of mouse natural killer cell and macrophage cytotoxic activities by enkephalins. *Pol J Pharmacol* 1995; 47:327-31; PMID:8616512.
- Mosnaim AD, Wolf ME, Maturana P, Mosnaim G, Puente J, Kucuk O, et al. In vitro studies of natural killer cell activity in post traumatic stress disorder patients. Response to methionine-enkephalin challenge. *Immunopharmacology* 1993; 25:107-16; PMID:8500983; [http://dx.doi.org/10.1016/0162-3109\(93\)90014-H](http://dx.doi.org/10.1016/0162-3109(93)90014-H).
- Stanojevic S, Mitic K, Vujic V, Kovacevic-Jovanovic V, Dimitrijevic M. The influence of stress and methionine-enkephalin on macrophage functions in two inbred rat strains. *Life Sci* 2007; 80:901-9; PMID:17157881; <http://dx.doi.org/10.1016/j.lfs.2006.11.019>.
- Vujic V, Stanojevic S, Dimitrijevic M. Methionine-enkephalin stimulates hydrogen peroxide and nitric oxide production in rat peritoneal macrophages: interaction of mu, delta and kappa opioid receptors. *Neuroimmunomodulation* 2004; 11:392-403; PMID:15467355; <http://dx.doi.org/10.1159/000080150>.
- Esche C, Makarenkova VP, Kost NV, Lotze MT, Zozulya AA, Shurin MR. Murine dendritic cells express functional delta-type opioid receptors. *Ann N Y Acad Sci* 1999; 885:387-90; PMID:10816671; <http://dx.doi.org/10.1111/j.1749-6632.1999.tb08695.x>.
- Makarenkova VP, Esche C, Kost NV, Shurin GV, Rabin BS, Zozulya AA, et al. Identification of delta- and mu-type opioid receptors on human and murine dendritic cells. *J Neuroimmunol* 2001; 117:68-77; PMID:11431006; [http://dx.doi.org/10.1016/S0165-5728\(01\)00313-7](http://dx.doi.org/10.1016/S0165-5728(01)00313-7).
- Bénard A, Boué J, Chapey E, Jaume M, Gomes B, Dietrich G. Delta opioid receptors mediate chemotaxis in bone marrow-derived dendritic cells. *J Neuroimmunol* 2008; 197:21-8; PMID:18486241; <http://dx.doi.org/10.1016/j.jneuroim.2008.03.020>.
- Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* 1997; 158:2723-30; PMID:9058806.

Sciences, Version 16.0) for Windows. All variables are presented as mean $\pm$ sd. Differences were evaluated by ANOVA for multiple groups and by the Student test for two groups using the Prism (Graph Pad Software). Tukey test used for post hoc analysis indicated significance when $p < 0.05$ by ANOVA. The results will be discussed later.

Disclosure of Potential Conflicts of Interest

The authors have no financial conflicts of interest with any party.

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14. Shan F, Xia Y, Wang N, Meng J, Lu C, Meng Y, et al. Functional modulation of the pathway between dendritic cells (DCs) and CD4+T cells by the neuropeptide: methionine enkephalin (MENK). *Peptides* 2011; 32:929-37; PMID:21335041; <http://dx.doi.org/10.1016/j.peptides.2011.01.033>.
15. Plotnikoff N, Wybran J. Methionine-enkephalin shows promise in reducing HIV in blood. *Am Fam Physician* 1989; 40:234; PMID:2773759.
16. Marotti T, Gabrilovac J, Rabatic S, Smejkal-Jagar L, Rocic B, Habersack H. Met-enkephalin modulates stress-induced alterations of the immune response in mice. *Pharmacol Biochem Behav* 1996; 54:277-84; PMID:8728569; [http://dx.doi.org/10.1016/0091-3057\(95\)02112-4](http://dx.doi.org/10.1016/0091-3057(95)02112-4).
17. Kraus J. Regulation of mu-opioid receptors by cytokines. *Front Biosci* 2009; 1:164-70.
18. Sharp BM. Multiple opioid receptors on immune cells modulate intracellular signaling. *Brain Behav Immun* 2006; 20:9-14; PMID:16364815; <http://dx.doi.org/10.1016/j.bbi.2005.02.002>.
19. Sulowska Z, Majewska E, Krawczyk K, Klink M, Tchórzewski H. Influence of opioid peptides on human neutrophil apoptosis and activation in vitro. *Mediators Inflamm* 2002; 11:245-50; PMID:12396476; <http://dx.doi.org/10.1080/096293502900000104>.
20. Menzebach A, Hirsch J, Hempelmann G, Welters ID. Effects of endogenous and synthetic opioid peptides on neutrophil function in vitro. *Br J Anaesth* 2003; 91:546-50; PMID:14504158; <http://dx.doi.org/10.1093/bja/aeg219>.
21. Stanojevic S, Vujic V, Mitic K, Kustrimovic N, Kovacevic-Jovanovic V, Miletic T, et al. Methionine-enkephalin modulation of hydrogen peroxide (H₂O₂) release by rat peritoneal macrophages involves different types of opioid receptors. *Neuropeptides* 2008; 42:147-58; PMID:18237778; <http://dx.doi.org/10.1016/j.npep.2007.12.004>.
22. Zagon IS, Donahue RN, Rogosnitzky M, McLaughlin PJ. Imiquimod upregulates the opioid growth factor receptor to inhibit cell proliferation independent of immune function. *Exp Biol Med* (Maywood) 2008; 233:968-79; PMID:18480416; <http://dx.doi.org/10.3181/0802-RM-58>.
23. Hucklebridge FH, Hudspeth BN, Lydyard PM, Brostoff J. Stimulation of human peripheral lymphocytes by methionine enkephalin and delta-selective opioid analogues. *Immunopharmacology* 1990; 19:87-91; PMID:2161808; [http://dx.doi.org/10.1016/0162-3109\(90\)90043-E](http://dx.doi.org/10.1016/0162-3109(90)90043-E).
24. Piva M, Moreno JI, Jenkins FS, Smith JK, Thomas JL, Montgomery C, et al. In vitro modulation of cytokine expression by enkephalin-derived peptides. *Neuroimmunomodulation* 2005; 12:339-47; PMID:16557034; <http://dx.doi.org/10.1159/000091127>.
25. Wang D, Meng J, Shan F. Immune system restore on terminal cancer patients by bio-peptide: Methionine Enkephalin (MEK). *J Microbiol* 2008; 28:39-41.
26. Akbar SM, Furukawa S, Hasebe A, Horiike N, Michitaka K, Onji M. Production and efficacy of a dendritic cell-based therapeutic vaccine for murine chronic hepatitis B virus carrier. *Int J Mol Med* 2004; 14:295-9; PMID:15254781.
27. Zagon IS, Verderame MF, Hankins J, McLaughlin PJ. Overexpression of the opioid growth factor receptor potentiates growth inhibition in human pancreatic cancer cells. *Int J Oncol* 2007; 30:775-83; PMID:17332915.
28. Slominski AT, Zmijewski MA, Zbytek B, Brozyna AA, Granese J, Pisarchik A, et al. Regulated proenkephalin expression in human skin and cultured skin cells. *J Invest Dermatol* 2011; 131:613-22; PMID:21191404; <http://dx.doi.org/10.1038/jid.2010.376>.
29. Cheng F, McLaughlin PJ, Zagon IS. Regulation of cell proliferation by the opioid growth factor receptor is dependent on karyopherin beta and Ran for nucleocytoplasmic trafficking. *Exp Biol Med* (Maywood) 2010; 235:1093-101; PMID:20705629; <http://dx.doi.org/10.1258/ebm.2010.010139>.
30. Avella DM, Kimchi ET, Donahue RN, Tagaram HR, McLaughlin PJ, Zagon IS, et al. The opioid growth factor-opioid growth factor receptor axis regulates cell proliferation of human hepatocellular cancer. *Am J Physiol Regul Integr Comp Physiol* 2010; 298:R459-66; PMID:19923357; <http://dx.doi.org/10.1152/ajp-regu.00646.2009>.
31. Gameiro SR, Caballero JA, Higgins JP, Apelian D, Hodge JW. Exploitation of differential homeostatic proliferation of T-cell subsets following chemotherapy to enhance the efficacy of vaccine-mediated antitumor responses. *Cancer Immunol Immunother* 2011; 60:1227-42; PMID:21544650; <http://dx.doi.org/10.1007/s00262-011-1020-8>.
32. Cany J, Tran L, Gauttier V, Judor JP, Vassaux G, Ferry N, et al. Immunotherapy of hepatocellular carcinoma: is there a place for regulatory T-lymphocyte depletion? *Immunotherapy* 2011; 3(Suppl):32-4; PMID:21524167; <http://dx.doi.org/10.2217/imt.11.29>.
33. Lombardi G, Sagoo P, Scotta C, Fazekasova H, Smyth L, Tsang J, et al. Cell therapy to promote transplantation tolerance: a winning strategy? *Immunotherapy* 2011; 3(Suppl):28-31; PMID:21524166; <http://dx.doi.org/10.2217/imt.11.42>.
34. Jing W, Yan X, Hallett WH, et al. Depletion of CD25+ T cells from hematopoietic stem cell grafts increases post-transplant vaccine-induced immunity to neuroblastoma. *Blood* 2011; 117: 6952-62.
35. Yan M, Jene N, Byrne D, Millar EK, O'Toole SA, McNeil CM, et al. Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers. *Breast Cancer Res* 2011; 13:R47; PMID:21521526; <http://dx.doi.org/10.1186/bcr2869>.
36. Munn DH. Indoleamine 2,3-dioxygenase, Tregs and cancer. *Curr Med Chem* 2011; 18:2240-6; PMID:21517755.
37. Schuler PJ, Böhrer V, Bölke E, Habermehl D, Matuschek C, Wild CA, et al. Dendritic cell generation and CD4+ CD25high FOXP3+ regulatory t cells in human head and neck carcinoma during radio-chemotherapy. *Eur J Med Res* 2011; 16:57-62; PMID:21463982; <http://dx.doi.org/10.1186/2047-783X-16-2-57>.
38. Walczak M, Regts J, van Oosterhout AJ, Boon L, Wilschut J, Nijman HW, et al. Role of regulatory T-cells in immunization strategies involving a recombinant alphavirus vector system. *Antivir Ther* 2011; 16:207-18; PMID:21447870; <http://dx.doi.org/10.3851/IMP1751>.
39. McLaughlin PJ, Zagon IS, Park SS, Conway A, Donahue RN, Goldenberg D. Growth inhibition of thyroid follicular cell-derived cancers by the opioid growth factor (OGF) - opioid growth factor receptor (OGFr) axis. *BMC Cancer* 2009; 9:369; PMID:19835629; <http://dx.doi.org/10.1186/1471-2407-9-369>.
40. Kuniyasu H, Luo Y, Fujii K, Sasahira T, Morioka Y, Tatsumoto N, et al. CD10 enhances metastasis of colorectal cancer by abrogating the anti-tumoural effect of methionine-enkephalin in the liver. *Gut* 2010; 59:348-56; PMID:19828468; <http://dx.doi.org/10.1136/gut.2009.178376>.
41. Zagon IS, Donahue RN, McLaughlin PJ. Opioid growth factor-opioid growth factor receptor axis is a physiological determinant of cell proliferation in diverse human cancers. *Am J Physiol Regul Integr Comp Physiol* 2009; 297:R1154-61; PMID:19675283; <http://dx.doi.org/10.1152/ajp-regu.00414.2009>.