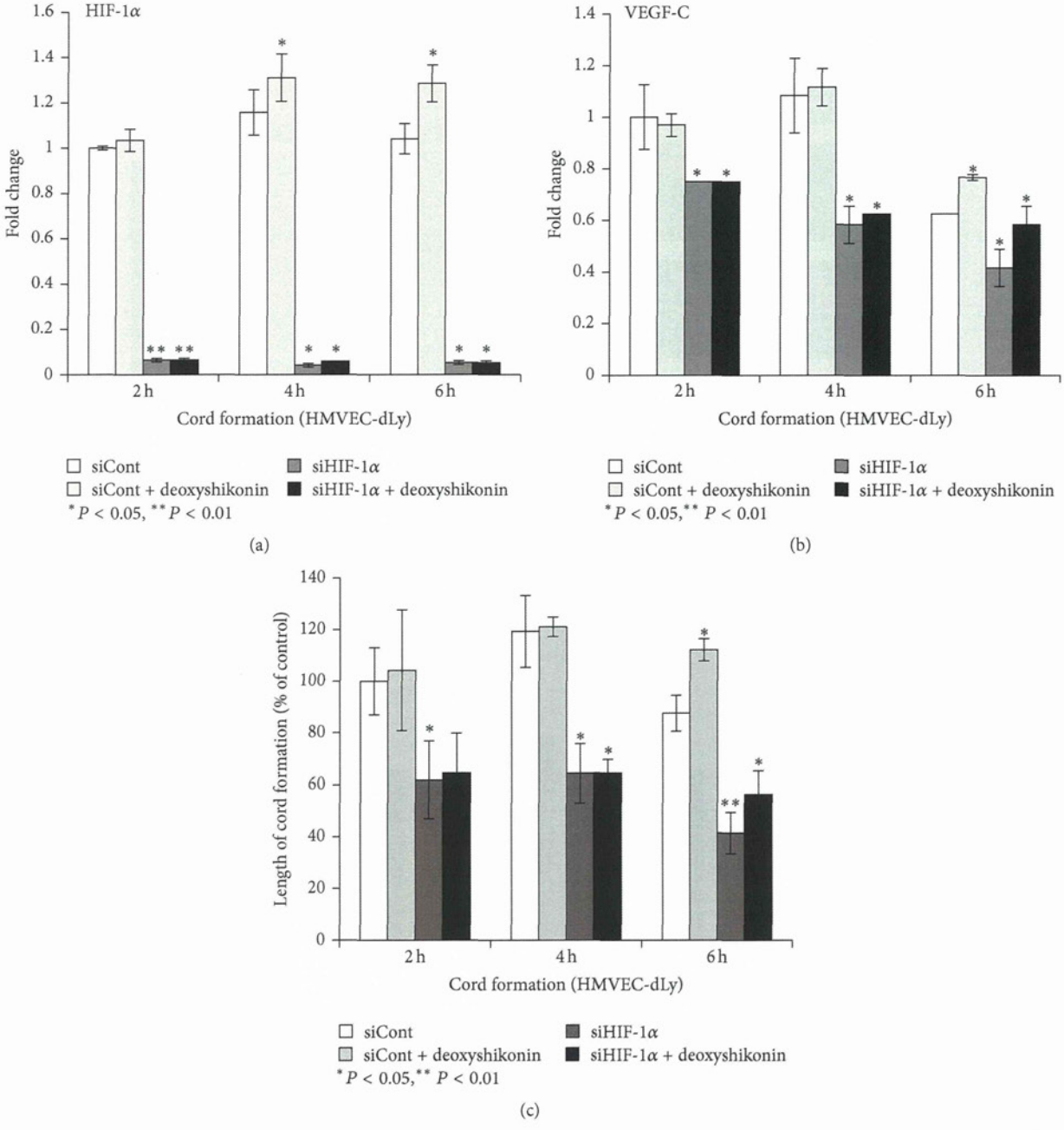

FIGURE 4: Deoxyshikonin regulates VEGF-C mRNA levels and cord formation of HMVEC-dLy via HIF-1α. After transfection with control siRNA or siRNA for HIF-1α, HMVEC-dLy cells were seeded on Matrigel and incubated with or without 0.8 μM deoxyshikonin for 2–6 h, and then cells were subjected to real-time PCR and cord formation assay. (a) HIF-1α mRNA levels. (b) VEGF-C mRNA levels. (c) Relative length of cord formations. Similar results were obtained in three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

human dermal microvascular endothelial cells (HMVEC-d). We continued to explore the possible mechanism by investigating the expression of important genes involved in cord formation networks of lymphatic endothelial cells after treatment with deoxyshikonin.

Previous reports found that deoxyshikonin has antifungal [23] and antitumor activities [24, 25], but there was no evidence of a lymphangiogenesis or angiogenesis effect. Shikonin and some derivative forms such as acetylshikonin, isobutyroylshikonin, and β-hydroxyisovalerylshikonin are already known to have an antiangiogenesis effect on an *in vivo* and *in vitro* model and the controlling molecules are known [9, 13, 16]; however, the effect of shikonin and its derivatives, including deoxyshikonin, on lymphangiogenesis has not been discovered.

The nontoxic dose of deoxyshikonin was confirmed (Figure 5(c)) and selected in a proliferation assay before performing the experiments. Our results show for the

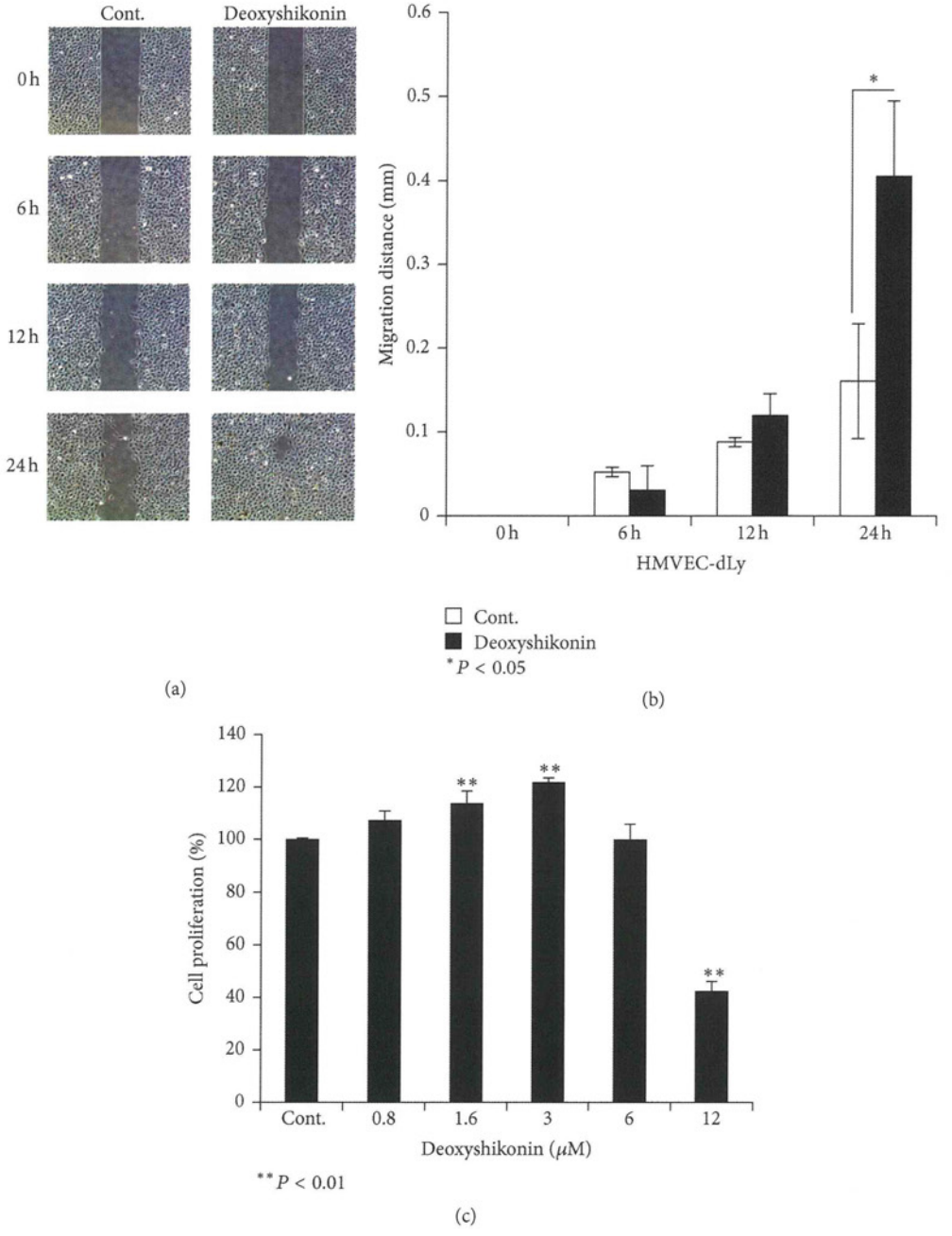

FIGURE 5: Deoxyshikonin promoted wound healing *in vitro* by inducing the HMVEC-dLy migration ability. After growing the HMVEC-dLy in culture inserts that create a cell-free gap, migration of cells to fill the gap was monitored at regular intervals. (a) Photograph of cell migration into the gap from 0 to 24 h in deoxyshikonin treatment and control group. (b) The migration ability of the cells was measured using Leica LAS EZ software and the migration distances calculated (mm). (c) The effects of deoxyshikonin on cell proliferation were determined by a proliferation assay and the data are plotted as percentages of control cell proliferation. Data are the mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

first time that deoxyshikonin has a potential to promote lymphangiogenesis and angiogenesis in an *in vitro* model, which interestingly showed opposite effects with shikonin (See Supplementary Figures 1(b) and 1(c) available online at http://dx.doi.org/10.1155/2013/148297). We showed for the first time that deoxyshikonin has a prolymphangiogenesis (Figures 2(a) and 2(b)) as well as a proangiogenesis effect *in vitro* (Figures 2(c) and 2(d)).

The processes of the cord formation of endothelial cells after seeding on Matrigel, which mimics the extracellular matrix [26], include the promotion of cell adhesion, survival, and migration, including cell proliferation for sprouting

and finding each other and maintaining the formation cord networks [27], and VEGFs such as VEGF-C, VEGF-A, and VEGF-D are widely known to induce lymphangiogenesis and angiogenesis *in vitro* and *in vivo* by enhancing these processes [28]. Generally, endothelial cells are the target, not the main source of VEGFs; however, it has been also demonstrated that human dermal microvascular endothelial cells themselves can express mRNA and release an amount of these growth factors [17, 28]. We found that deoxyshikonin significantly increased the expression of VEGF-C mRNA and VEGF-A mRNA in HMVEC-dLy but had no effect on VEGF-D (Figure 3(b)). The increase of VEGF-C and VEGF-A correlated with the significant induction of the cord formation of HMVEC-dLy at the time of deoxyshikonin treatment (Figure 3(a)). Endothelium-derived VEGF can induce neovascularization through proliferation, and increase the migration of dermal microvascular cells [29]. The increase of VEGF-C and VEGF-A mRNA expression after deoxyshikonin treatment could proceed to protein products, be secreted, and then interact with specific membrane receptors of endothelial cells displaying tyrosine kinase activity [17]. Binding of VEGF-A with VEGFR-2 and binding of VEGF-C with VEGFR-2 and VEGFR-3 promote the cord formation of HMVEC-dLy on Matrigel (Figure 3(a)). Interestingly, the results (Figure 3(b)) show that the mRNA levels of VEGF-C after deoxyshikonin treatment were high when compared with VEGF-A. As VEGF-C acts as a key growth factor in physiological lymphangiogenesis and was found to promote the activation of VEGF-3, a specific receptor expressed in lymphatic endothelium [18, 19], we focused our study on the mechanism of deoxyshikonin on lymphangiogenesis *in vitro*.

HIF-1 is an oxygen-regulated transcriptional factor that plays a role in tumor lymphangiogenesis, wound healing and inflammation by regulating the lymphatic expression of VEGF-C [7, 20, 21]. In addition, HIF-1-mediated pathways also promote or repress the transcription of a broad range of genes that are involved in maintaining biological homeostasis, such as influencing metabolic adaptation, the innate immune response, cell survival, and apoptosis [4, 5]. In hypoxia, HIF-1α protein persists and the HIF-1α/β complex stimulates VEGF release in almost all cell types. Under normoxia, HIF-1α protein is subjected to ubiquitin-dependent degradation [4]; however, HIF-1α is also expressed and functions in response to stimulation by several growth factors by the mechanism different from the hypoxic condition [5].

In this study, we performed experiments under normoxic conditions. For the first time we found that deoxyshikonin regulated HIF-1α at transcriptional, posttranscriptional, and functional levels (Figures 3(c), 3(d), and 3(e)) during cord formation of HMVEC-dLy (Figure 3(a)). Similar recent reports mentioned that several nonhypoxic effectors and signaling pathways have been proven to enhance HIF-1α levels through the activation of regulative mechanisms distinct from protein stabilization. Some of these stimuli also regulate HIF-1α at the transcriptional, posttranscriptional, or translational level or additionally influence posttranslational modifications, including the functions of HIF-1α protein [4, 5]. For example, lipopolysaccharides (LPS) and cytokines activate the nuclear factor-κB (NF-κB) signaling pathway promoting HIF-1α transcription [30], whereas some growth factors such as epithelial growth factors (EGF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor (IGF) enhance the translation of HIF-1α protein [31]. In addition, loss of function of tumor suppressors (such as p53, PTEN, and VHL) and gain of function of oncogenes (such as AKT, MYC, mTOR, PI3 K, RAF, and RAS) also regulate different steps that lead to the activation of HIF function [31, 32].

Deoxyshikonin might contribute to the signaling pathways mentioned above, enhance HIF-1α mRNA/protein expression and activate nuclear translocation (Figures 3(c), 3(d), and 3(e)); however, we did not prove the effect of deoxyshikonin on the signal transduction pathway in this study. Once in the nucleus, deoxyshikonin could promote HIF-1α and HIF-1β subunit interaction and bind to specific DNA sequences targeted by HIF, known as hypoxia response elements (HREs), which are composed of 5′-RCGTG-3′, leading to the stimulation of VEGF release, especially VEGF-C, which induced cord formation of HMVEC-dLy (Figures 3(a) and 3(b)). Successful suppression of the HIF-1α gene using siRNA transfection confirmed that HIF-1α regulated VEGF-C mRNA expression and the cord formation ability of HMVEC-dLy on Matrigel (Figure 4).

Although VEGF mRNA, including VEGF-C mRNA expression, can be upregulated by HIF-1, several transcription factors such as AP-1, Sp-1, and NF-κB also induce VEGF expression by binding to the promoter to initiate and activate the transcription of the VEGF gene directly [6]. We also proved this by using deoxyshikonin treatment in HIF-1α knockdown cells and found that deoxyshikonin could not recover VEGF-C mRNA expression and the cord formation of HMVEC-dLy back to the control level but only slightly increased when compared with HIF-1α knockdown cells alone (Figure 4). This result indicated that deoxyshikonin induced VEGF-C mRNA expression and the cord formation of HMVEC-dLy, mainly via HIF-1α-dependent regulation, and may also contribute to HIF-1α-independent regulation; however, the details of these mechanisms still need to be further investigated.

The promotion of lymphatic vessel generation improved wound function to maintain normal tissue pressure by draining protein-rich lymph from the interstitial space and facilitate the delivery of cells that mediate the immune response [22]. In this study we proved that deoxyshikonin promoted lymphangiogenesis (Figure 2) and also wound healing *in vitro* by facilitating the migration of HMVEC-dLy into the wound gap (Figure 5), which indicated that deoxyshikonin could be developed for use in wound-healing treatment. However, wound healing is a complicated biological process as it involves the interactions of multiple cell types, various cytokines, growth factors, their mediators, and extracellular matrix proteins [11], and the details of deoxyshikonin in wound healing require further proof *in vitro* and also *in vivo*.

In conclusion, we discovered a new effect of deoxyshikonin, which is included in shiunko as a typical Kampo drug ointment used for the treatment of wound healing in Japan, that enhanced cord formation of HMVEC-dLy via HIF-1α-controlled VEGF-C mRNA regulation and also promoted

wound healing in an *in vitro* model. This finding may offer new therapeutic options for using deoxyshikonin compounds that modulate HIF-1α and VEGF-C under nonhypoxic conditions in wound healing and other lymphatic diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

This study was supported by a Grant-in-Aid for Scientific Research (C) (no. 22501042).

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Bioorganic & Medicinal Chemistry Letters 22 (2012) 3001–3005

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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Synthesis and evaluation of 1-(substituted)-3-prop-2-ynylureas as antiangiogenic agents

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ARTICLE INFO

Article history:
Received 12 September 2011
Revised 20 January 2012
Accepted 10 February 2012
Available online 22 February 2012

Keywords:
1-(Substituted)-3-prop-2-ynylurea
Cytotoxic agents
Tyrosine kinase inhibitors
EGFR kinase inhibitor
Antiangiogenic agent

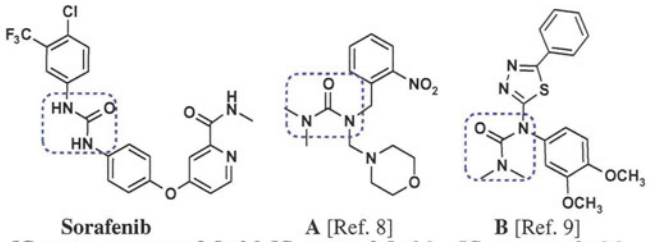
ABSTRACT

Novel urea derivatives of alkynes have been designed, synthesized, and evaluated as potential cancer therapeutics leads. The most active 1-((3-chloromethyl)phenyl)-3-prop-2-ynylurea (**1**) exhibited cytotoxic effect against HELA and MCF-7 cell lines with IC_{50} values of 1.55 μM and 1.48 μM, respectively. Further investigation on tube formation assay in human vein umbilical cells (HUVEC) demonstrated that **1** and methyl 4-(3-(3-ethynylureido)benzyloxy) benzoate (**6**) possess antiangiogenic activity, with minimum effective dose of 25 nM (for **1**) and 6.25 μM (for **6**). The ED_{50} of **1** and **6** were found to be 0.26 μM and 17.52 μM, respectively. The results from in vitro tyrosine kinase assay indicated the EGFR inhibition of **1** over other kinases (VEGFR2, FGFR1 and PDGFRβ). The cytotoxicity of **1** against EGFR overexpressing cell line A431 (IC_{50} 36 nM) was comparable to that of erlotinib. The binding mode of **1** from docking simulation in the EGFR active site revealed that the urea motif formed hydrogen bonding with Lys745, Thr854 and Asp855 in hydrophobic pocket of EGFR. Compound **1** is considered as a potential lead for further optimization.

Protein tyrosine kinases (TKs), a family of cell signaling proteins that regulate inter- and intracellular communications, have been implicated in cancer development. Two classes of TKs, categorized by structures, functions and locations, are receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). These TKs play an important role in regulation of cell growth, proliferation, differentiation, survival and metabolism.[1,2] RTKs such as vascular endothelial growth factor receptor-2 (VEGFR2), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor-1 (FGFR1), and platelet-derived growth factor receptor (PDGFR) play a key role in angiogenesis, the sprouting process of capillaries from pre-existing vessels. These RTKs are important targets in antiangiogenic drug development. Inhibition of these enzymes can lead to suppression of both cell proliferation and angiogenesis and may become a viable strategy in cancer treatment.[3]

Urea derivatives demonstrate diverse array of biological and pharmacological activities, such as antibacterial, antifungal, anti-inflammatory, antiangiogenic and antiproliferative properties.[4–6] Sorafenib is an example of an anti-proliferative urea agent (Fig. 1).[7–9] It is a tyrosine kinase inhibitor (VEGFR and PDGFR) which was approved by the US FDA for advanced renal cancer.[10]

The 1-(substituted)-3-prop-2-ynylureas, terminal alkynes bearing urea motif, were firstly designed in our lab as an in-house library for the preparation of 1,4-disubsituted-1,2,3-triazole derivatives by copper-catalyzed azide alkyne cycloaddition (CuAAC) reaction.[11]



Sorafenib IC_{50}(MDA-MB-231) = 2.5 μM

A [Ref. 8] IC_{50} (KB) = 2.5 μM, IC_{50} (K562) = 0.4 μM

B [Ref. 9] IC_{50} (K562) = 3 μM

Figure 1. Substituted ureas acting as antiproliferative agents. MDA-MB-231, KB and K562 are human breast cancer cell line, human carcinoma of the nosopharynx and human erythroleukemia cell line, respectively.

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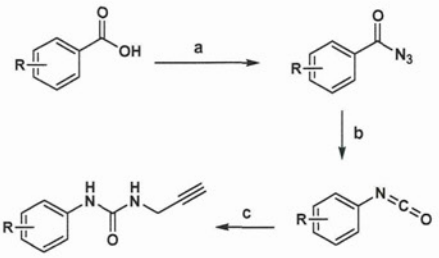
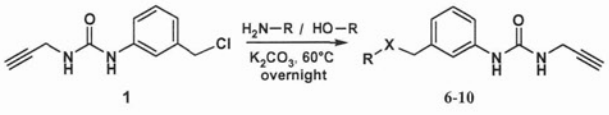
doi:10.1016/j.bmcl.2012.02.029

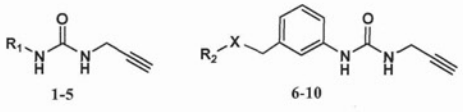


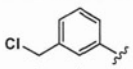
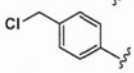
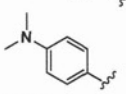
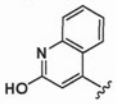
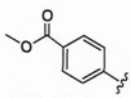
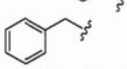
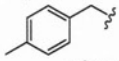
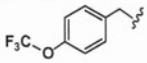
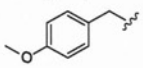
Scheme 1. General synthesis of urea compounds **1–5**. Reagents and conditions: (a) DPPA, TEA, toluene or dioxane, rt, 30 min; (b) toluene or dioxane, 100 °C, 1 h; (c) propargylamine, rt, 30 min.



Scheme 2. General synthesis of urea compounds **6–10**.

Table 1
Yields and melting points of the synthesized 1-(substituted)-3-prop-2ynylureas



Compoundd	R_1	X	R_2	% Yield	Mp (°C)
1		–	–	21.48	158–160
2		–	–	10.01	132–134
3		–	–	50.27	178–180
4		–	–	72.48	122–124
5		–	–	10.53	246–248
6	–	O		38.12	179–181
7	–	NH		12.23	68–72
8	–	NH		25.53	119–120
9	–	NH		15.34	112–114
10	–	NH		34.87	99–101

The synthesized triazole-based ureas, as well as the azide and alkyne precursors, were screened for their cytotoxic effects against HELA and MCF-7 cell lines. 1-(3-Chloromethyl)-3-prop-2-ynylurea **1** showed cytotoxic effect against HELA and MCF-7.

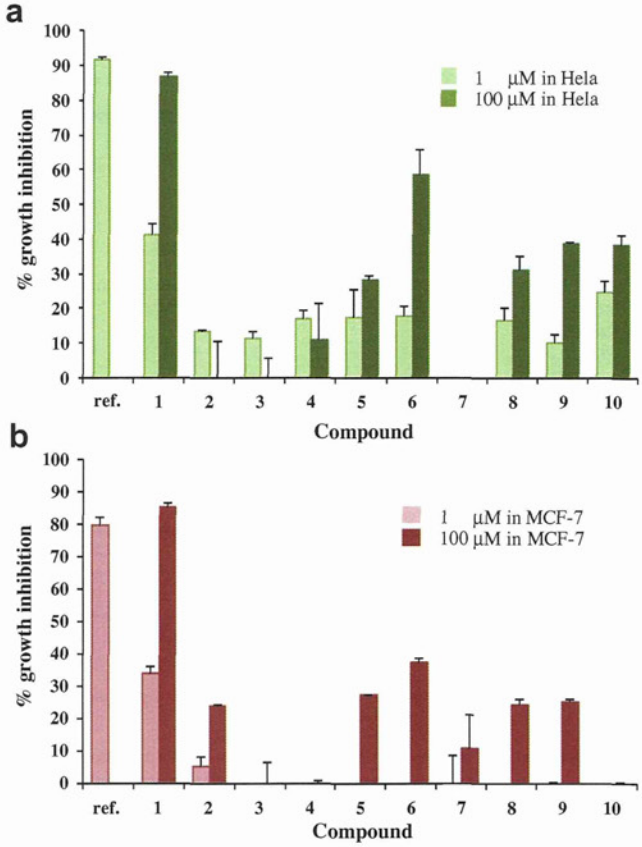
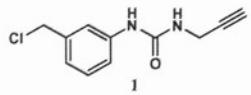


Figure 2. In vitro cytotoxicity effect of 1-(substituted)-3-prop-2-ynylureas at 1 μM and 100 μM against HELA (a) and MCF-7 (b). Doxorubicin at 1 μM was used as reference compound, $n = 3$.



A family of 26 of 1-(substituted)-3-prop-2-ynylureas were next designed by molecular modeling to increase the potency. Flexible ligands were docked to a grid representation of VEGFR2 model derived from crystal structure of the VEGFR2 tyrosine kinase domain in complex with a pyridyl-pyrimidine benzimidazole inhibitor (PDB: 3EWH)[12] using AutoDock4.2[13] (docking experiment and data of all 26 compounds are shown in Supplementary data). Ten compounds were selected based on free energy of binding, hydrogen bond interaction and visual inspection and synthesized and preliminary screened for their cytotoxic effects toward two types of human cancer cell lines, HELA and MCF-7.

Compounds **1–5** were prepared by the reaction between various phenyl isocyanates and propargylamine. The synthetic route is illustrated in Scheme 1.[14,15] Various phenyl isocyanates were prepared in situ via Curtius rearrangement of corresponding acyl azides before reacting with propargylamine to give 1-(substituted)-3-prop-2-ynylureas **1–5**. Briefly, acyl azides were prepared from the corresponding benzoic acids with diphenylphosphoryl azide (DPPA) in the presence of triethylamine in an appropriate solvent. Then, acyl azides were heated at 100 °C to achieve the corresponding isocyanates that were subsequently reacted with propargylamine yielding the desired compounds **1–5**, unoptimized yield: 10–72% (Supplementary data). Compounds **6–10** were synthesized by displacement of chloride of compound **1** with phenols or benzylamines in the presence of K_2CO_3 in one step fashion[16] as illustrated in Scheme 2 (unoptimized yield: 17–80%, synthesis

Table 2
IC_{50} values of the cytotoxicity against HELA, MCF-7

Compound	IC_{50}[a] (μM)	
	HELA	MCF-7
1	1.55	1.48
6	12.26	74.87
7	NA[b]	343.48
8	329.10	49.89
9	339.00	282.90
10	391.50	194.90
Doxorubicin	0.19	0.06

[a] Mean values of three independent experiments are reported.
[b] No activity.

Table 3
IC_{50} against the HUVEC

Compound	IC_{50}[a] (μM)	
	Primary	EA.hy926
1	20	10.54
6	100	78.23
7	50	191.72

[a] Mean values of three independent experiments are reported.

details are shown in Supplementary data). All synthesized compounds were displayed in Table 1.

The synthesized 1-(substituted)-3-prop-2-ynylureas **1–10** were then tested for their cytotoxic effects at 1 and 100 μM against HELA and MCF-7 using MTT assay[17] (Supplementary data). Doxorubicin was used as reference. The compounds that showed growth inhibition over 30% at 100 μM against either cancer cell line were selected for IC_{50} determination.

The initial screening results at two-point concentration of compounds **1–10** were shown in Figure 2. Concentration dependent cytotoxic effects of the compounds in this series against HELA and MCF-7 were observed. Although all compounds demonstrated poor cytotoxic activities comparing to doxorubicin, compound **1** exhibited substantial cytotoxic effect at 100 μM against both HELA and MCF-7 (growth inhibitions of 86.86% and 85.16% for HELA and MCF-7, respectively). Data from cytotoxicity assay of compounds **1–5** suggested that both size and position of substituents on 1-(substituted)-3-prop-2-ynylurea greatly affected on cytotoxic properties of the compounds in this series. Substituents on *para*-position of 1-(phenyl)-3-prop-2-ynylurea decreased cytotoxic effect regardless of the size of substituents as observed in compounds **2** and **3**. Bulky substituent that is, fused ring at position 1 of urea motif (compound **5**) also suppressed cytotoxic potency. The extended structure of compound **1** by displacement of chloride moiety with various phenyl or benzyl via ether- or amine-linker (compounds **6–10**) did not enhance anticancer activity as anticipated, even deteriorated. These emphasized the influence of size of the substituent on phenyl ring at position 1 of urea motif on the cytotoxicity.

Compounds **1**, **6** and **8–10** showed % growth inhibition over 30% at 100 μM against either or both HELA and MCF-7 were selected for the determination for IC_{50}. Among the compounds in this series, compound **1** is most potent compound showing cytotoxic effect on both HELA and MCF-7 with IC_{50} of 1.55 μM and 1.48 μM, respectively (Table 2).

Compounds **1** and **6** showing the best activity against two cancer cell lines were further evaluated for their antiangiogenic effects by tube formation assay, regardless of the considerably high cytotoxic IC_{50} in μM level. Compound **7** with poor activity was included in the assay for comparison. Tube formation assays were performed at non-cytotoxic doses of each compound against the human vein umbilical cell line (HUVEC) (Supplementary data). To identify non-cytotoxic doses for tube formation assay, the in vitro cytotoxicity of compounds **1**, **6** and **7** against HUVEC were performed by MTT assay in the similar fashion as of HELA and MCF-7. IC_{50} values of selected compounds against HUVEC were listed in Table 3.

Tube formation assays of compounds **1**, **6** and **7** were performed. Comparing with control, both compounds **1** and **6**

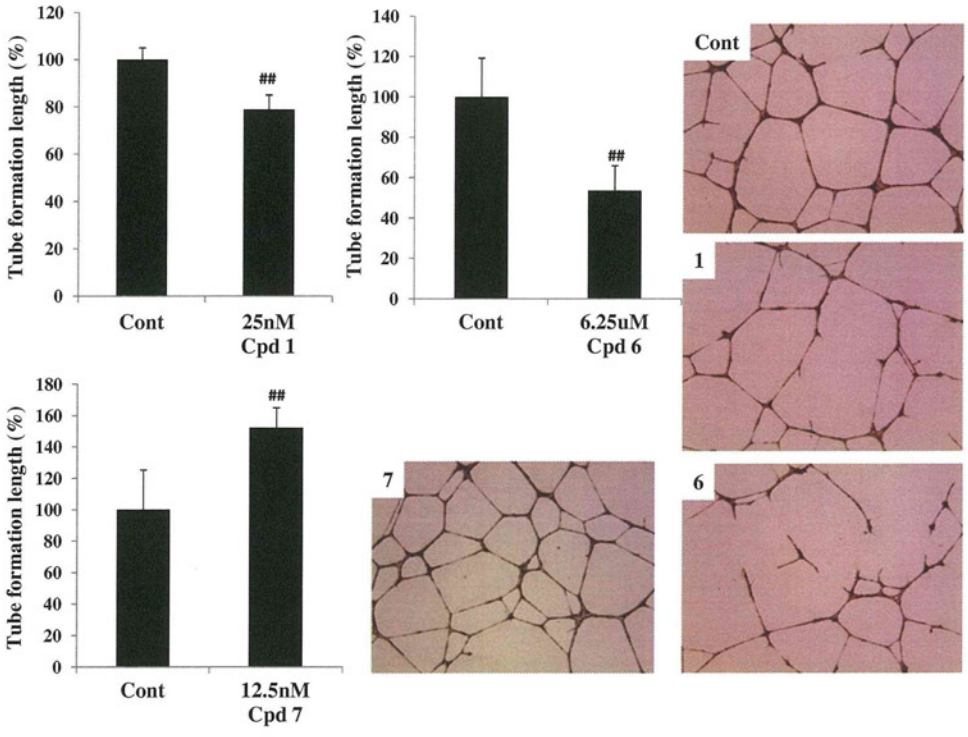


Figure 3. Effect of compounds **1**, **6** and **7** on tube formation comparing with control, $n = 3$, $^{**}p < 0.01$.

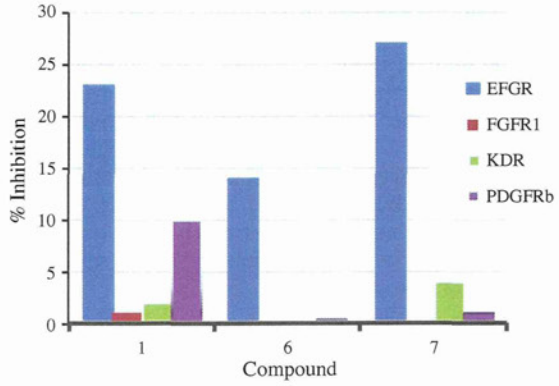


Figure 4. Kinase inhibitory profile at 1 μM against EGFR, FGFR1, VEGFR2 and PDGFRβ tyrosine kinases, % inhibition of erlotinip at 1 μM against EGFR was 98%.

significantly suppressed tube formation of HUVEC. The half maximal effective concentration (EC_{50}) of compounds **1** and **6** against tube formation were found to be 0.26 μM and 17.52 μM, respectively. The observed results from tube formation assay indicated that compounds **1** and **6** possessed antiangiogenic properties against HUVEC. The IC_{50}:ED_{50} ratios for HUVEC of compounds **1** and **6** were 77 and 6, respectively. The antiangiogenic ED_{50} dose of compound **1** was 77 times lower than its cytotoxic IC_{50} dose while that of compound **6** was only 6 times. Apparently, compound **1** did not only possess more potent antiangiogenic activity but also showed safer profile than compound **6**. The minimum effective doses were also determined at the non-cytotoxic dose, compounds **1** and **6** significantly suppressed tube formation of HUVEC at 25 nM and 6.25 μM with 21.18% and 46.47% inhibition, respectively (Fig. 3). Interestingly, compound **7** at non-cytotoxic dose (12.5 nM) showed the increase in tube formation, this compound is currently under investigation for its molecular mechanism.

Compounds **1** and **6** exhibiting significant antiangiogenic activity were evaluated for their effects on tyrosine kinases involving in angiogenesis. In vitro kinase assay of compounds **1** and **6** at 1 μM against VEGFR2, FGFR1, EGFR and PDGFRβ were performed. The levels of phosphorylation of the tyrosine kinase-specific ligand peptides[18–21] at 1 μM of test compounds were measured (Supplementary data). The kinase inhibition profile was displayed in Figure 4.

The tested compounds predominantly inhibited EGFR tyrosine kinase more than other kinases. At 1 μM, compound **1** exhibited significant activity against EGFR and PDGFRβ while compounds **6** and **7** inhibited EGFR tyrosine kinase only. The better cytotoxicity of compound **1** may be due to its ability to inhibit two kinases, EGFR and PDGFRβ.

Since compounds **1** and **6** inhibited EGFR more than the intended VEGFR2, docking of these compounds into ATP binding site of EGFR was performed using AutoDock4.2 to simulate a binding model. The EGFR model was derived from crystal structure of erlotinib bound EGFR obtained from RCSB (PDB: 1M17).[22] Dock poses

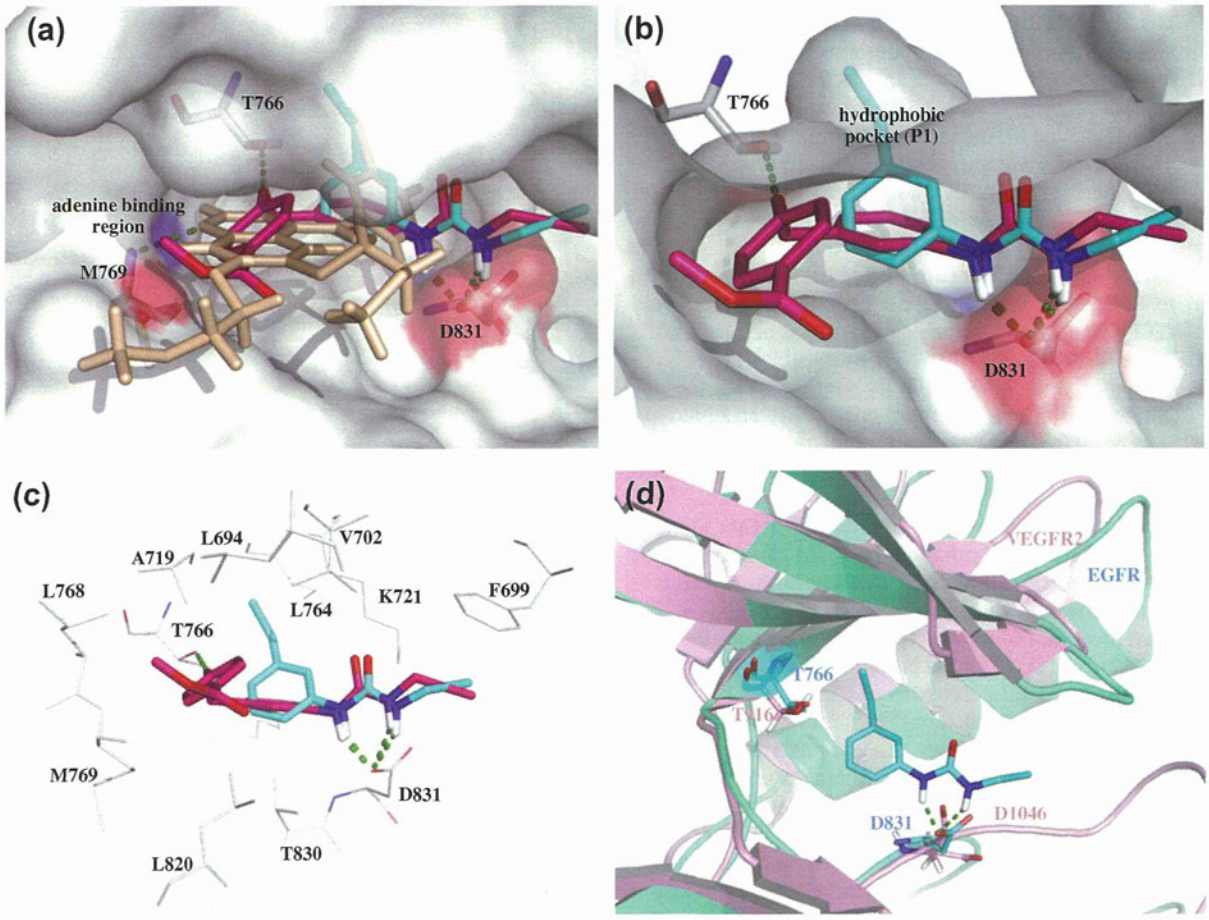


Figure 5. (a) Overlay of binding models of 1-(substituted)-3-prop-2-ylnylurea **1** (cyan), **6** (magenta) and erlotinib (wheat) in kinase domain of EGFR, (b) Overlay of **1** and **6** and their H-bond interactions in the EGFR active site (green dot line), **1** occupied back cavity of ATP binding site of EGFR (P1), (c) binding modes of **1** and **6** displaying interacted amino acid residues, (d) overlay binding model of compound **1** in kinase domain of EGFR (blue) and VEGFR2 (pink), key amino acid residues for H-bond interactions (observed in compounds **1** and **6**) in EGFR (T766 and D831) and in VEGFR2 (T916 and D1046) were illustrated.

Table 4
IC_{50} against A431 cell lines

Compound	IC_{50} (nM)
1	36.00
6	215.50
7	318.00
Erlotinib	55.50

of these compounds were compared with crystal pose of erlotinib as shown in Figure 5a.

EGFR docking results showed that 1-(substituted)-3-prop-2-ynylureas **1** and **6** partially occupied the ATP binding pocket of EGFR whereas erlotinib extensively occupied EGFR kinase domain (Fig. 5a). The partial occupancy may explain the observed low potency in kinase inhibition of these compounds. The 3-chloromethylphenyl substituent of **1** penetrated deeply in the back cavity of the ATP which was a poorly conserved hydrophobic area of the ATP binding site of EGFR.[23] This part of structure was surrounded by the hydrophobic side chains of Phe699, Val702, Ala719, Leu764, Leu820 and the hydrophobic parts of Lys721, Thr766 and Thr830. The urea motif established two hydrogen bonds with the carboxylate of conserved Asp831, a component of DFG motif in the beginning of the activation loop of EGFR which involved in Mg-ATP binding[24] (Fig. 5b). The occupied location of compound **6** was similar to those of **1**, two hydrogen bonds between both *H*N of urea and *C*OOH of Asp831, and an extra hydrogen bond between oxygen atom of ether and O*H* of Thr766, gatekeeper residue of EGFR (Fig. 5b). In addition to Thr766 and Asp831, amino acid residues in hydrophobic pocket that involved hydrophobic interaction included Leu694, Phe699, Val702, Ala719, Lys721, Leu764, Leu768, Met769, Leu820 and Thr830 (Fig. 5c). The binding mode cannot explain the higher potency of **1** over **6** since **6** showed more interacted hydrogen bonds over **1**. The overlay binding mode of **1** in kinase domain of EGFR and VEGFR2 was displayed in Figure 5d. Compound **1** also occupied back cavity of VEGFR2 and was surrounded by the hydrophobic side chains of Val848, Phe1047, Leu1049 and the hydrophobic part of Arg842, Lys868, Asp1046 and Asp1052 (data not shown). The observed hydrogen bond interactions cannot explain the selectivity of **1** against EGFR over VEGFR2 since **1** located in the similar region and bound to DFG motif of both kinases (Asp831 of EGFR and Asp1046 of VEGFR2). However, sequence alignment between EGFR (PDB: 1M17) and VEGFR2 (PDB: 3EWH) by Needle (EMBOSS)[25] showed 28.6% identity and 44.4% similarity suggested that the difference in the hydrophobic component of back cavity observed between EGFR and VEGFR2 might be the key factor controlling the selectivity of **1** and **6** against EGFR over VEGFR2; which appeared to be consistent with the reported notion.[23]

As compounds **1** and **6** were found to inhibit different kinases, especially EGFR, the effect on human epidermoid carcinoma cells A431, EGFR overexpressing cell lines[26–29] was performed (Supplementary data) and the IC_{50} values were reported in Table 4. The IC_{50} values in nM level for EGFR overexpressing A431 cells demonstrated the significance of EGFR kinase inhibition as those for HUVEC were in μM level. The cellular cytotoxicity of **1** was comparable to erlotinip despite of moderate EGFR kinase inhibition, it was possibly due to its ability to inhibit two kinases, EGFR and PDGFRβ.

In summary, a series of novel 1-(substituted)-3-prop-2-ynylureas based on structural modification of compound **1** were prepared to increase the binding capability. The structural modifications of **1** with extended aromatic side chains to increase the binding capability did not enhance tyrosine kinase inhibition nor antiproliferative as expected. Compounds **1** and **6** were cytotoxic against human cancer cells and demonstrated antiangiogenic effect in vitro. In EGFR overexpressing cell line (A431), the cytotoxicity of compound **1** was in nM level comparable to that of erlotinip. The binding mode of **1** from EGFR docking simulation demonstrated that the smaller in size of **1** facilitates and accommodates the hydrogen bond formation in the active sites of the receptors as evidenced by the inhibition of two types of tyrosine kinases (EGFR and PDGFRβ). The urea motif in the compound plays an important role in hydrogen bond interaction with amino acid residues in the active binding site. Terminal alkyne containing urea motif can be considered as new scaffold for further optimization of a potential cancer therapeutic lead.

Acknowledgments

This work was supported by the Royal Golden Jubilee Ph.D. Program (RGJ Grant No. PHD/0229/2547) funded by Thailand Research Fund (TRF) and the commission of Higher Education Thailand (CHE-RG 2551). The authors thank Dr. Benjamin Fraser and Dr. Neil P. Grimster for their valuable advice in some synthesis reactions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.02.029.

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ELSEVIER

Immune regulation and monitoring at the epithelial surface of the intestine

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The intestinal enterocytes and other epithelial cells create physical barriers, including tight junctions and mucus layers. These cells also actively transport antibodies across the epithelium and simultaneously produce antimicrobial peptides and enzymes. These functions maintain intestinal homeostasis by allowing the selective absorption of nutrients and simultaneously preventing pathogenic infections. Recent evidence has revealed that both host-derived factors (e.g., cytokines) and gut environmental factors (e.g., commensal bacteria, dietary materials, and their metabolites) regulate the physical and immunological functions of the epithelium. Understanding the interactions between host cells and these environmental factors should help us to develop new strategies to prevent and treat immune diseases of the intestine.

The surface of the gastrointestinal tract is covered by a single layer of epithelium that separates the outside world from interstitial tissues. The intestinal epithelium is mainly composed of absorptive enterocytes (ECs) but also includes enteroendocrine, goblet, and Paneth cells [1]. Cross-communication among these cells enables the selective absorption of nutrients while simultaneously preventing the penetration of antigens and pathogens. The defense against pathogenic materials is at least partly achieved by the physical barriers of the epithelium, which include tight junctions and mucus layers. A large number of pathogens disrupt these barriers to access deeper tissues for dissemination [2,3]. The barriers also contribute to the establishment and maintenance of mucosal homeostasis. Indeed, a leaky intestinal barrier is one of the characteristics of chronic intestinal inflammatory diseases, such as inflammatory bowel disease and celiac disease [4,5].

Intestinal tissues also show intense immunological activity, and ECs contribute to the intestinal immune system by transporting and processing antibodies and associated antigens, by producing immunologically functional molecules, and by interacting with immunocompetent cells in the intestine [6]. Accumulating evidence has revealed that both host-derived factors (e.g. cytokines) and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites) engage in molecular crosstalk with the intestinal epithelium and affect intestinal barrier function and immune responses [7,8]. In this review, we focus on the immunological functions of ECs in the intestine and their regulation by commensal bacteria and dietary materials.

Physical barriers at the intestinal epithelium

Tight junctions

ECs provide a physical barrier to prevent the paracellular transport of luminal antigens and pathogens. Tight junctions are multifunctional complexes that are crucial for the maintenance of barrier integrity because they form a seal between adjacent ECs [9]. The tight junction regulates the absorption of nutrients, ions, and water while preventing the entry of pathogens into the host.

Tight junctions are composed of numerous interacting cellular proteins, including claudin, occludin, and zonula occludens (ZO) proteins (Fig. 1). Claudin and occludin are transmembrane proteins that seal the paracellular space between adjacent ECs. Among

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1359-6446/06/$ - see front matter http://dx.doi.org/10.1016/j.drudis.2012.08.001

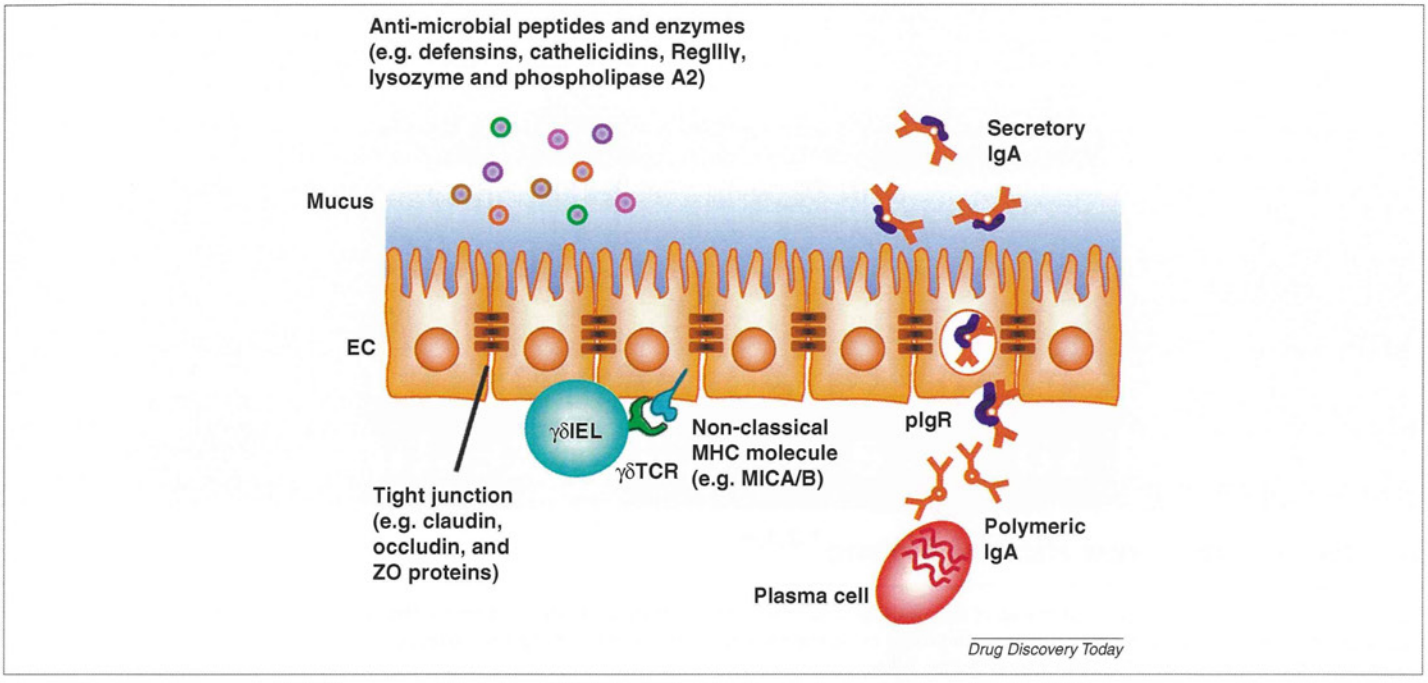

FIGURE 1

Physical and immunological barriers mediated by ECs. ECs (including Paneth cells) produce several molecules that create physical barriers in the intestine. They also produce antimicrobial peptides and enzymes, such as defensins, cathelicidins, RegIIIγ, lysozyme, and phospholipase A2 to kill the bacteria and establish a mucus layer to prevent bacterial attachment to the ECs. Tight junctions among ECs prevent bacterial penetration between the cells. ECs also have immunological functions. They express polymeric immunoglobulin receptor (pIgR), which binds and transports polymeric IgA produced from plasma cells into the intestinal lumen. ECs exposed to stresses (e.g. infection or cancer) express non-classical MHC molecules (e.g. MICA/B). MICA/B acts as a ligand for γδ T cell receptors, which are uniquely expressed on intraepithelial lymphocytes (γδIELs). *Abbreviations*: EC, enterocytes; MHC, major histocompatibility complex; ZO, zonula occludens.

the various types of claudins, claudin-1, -2, -3, -4, -5, -7, -8, -12, -15, -18, -20, and -23 are expressed in the intestinal epithelium [10,11]. ZO proteins are adaptors that connect transmembrane proteins; in particular, ZO-1 interacts with the claudin proteins and with F-actin in the intestinal ECs [12,13].

The physical barriers created by ECs are at least partly regulated by the immunological stimulation provided by commensal bacteria and dietary materials. Indeed, commensal and probiotic bacteria, their metabolites, food extracts, and dietary materials (e.g. fatty acids, polysaccharides, and flavonoids) have been shown to promote intestinal barrier integrity by increasing the expression of tight junction proteins [10].

Mucus

The mucus layer has been recognized as an important component in the intestine (Fig. 1). Mucin 2 (MUC2), a large glycoprotein characterized by variable O-linked glycans, is abundantly expressed by goblet cells located in the intestinal epithelium [14]. Generally, mucus can be divided into two layers. Although both layers have similar protein composition, the outer mucus layer is loose, whereas the inner mucus layer adheres firmly to the surface of the ECs. The firm mucus in the inner layer is an efficient barrier against pathogens [15]. In addition to the physical and biological barrier function of mucus, mucus also ensures the concentration of antimicrobial peptides and IgA antibodies at the surface of ECs. As similar to tight junctions, mucus expression is regulated by commensal bacteria, and the mucus layer of germ-free mice is thicker than that of specific pathogen-free mice [15].

Production of antimicrobial molecules at the epithelium

Antimicrobial peptides

The epithelium also secretes a variety of antimicrobial peptides [e.g. defensins, cathelicidins, and RegIIIγ (Fig. 1)]. The production of these peptides is mainly mediated by ECs and Paneth cells [16]. Paneth cells reside at the base of the crypt regions of the intestine, where they constitutively produce α-defensins. This does not require bacterial stimulation, because Paneth cells produce normal amounts of α-defensin in germ-free mice [17]. By contrast, ECs require microbial stimulation for the production of β-defensins [16]. ECs also produce cathelicidin, the expression of which is regulated by short-chain fatty acids produced when polysaccharides are metabolized by fermenting bacteria [18]. Both defensins and cathelicidin are cationic small peptides that exhibit antimicrobial activity by damaging and permeabilizing the bacterial cell membrane by pore formation [19].

RegIIIγ is a C-type lectin produced by ECs and Paneth cells in the ileum, where it kills Gram-positive bacteria by binding to surface-exposed carbohydrate moieties of peptidoglycans [20]. Commensal bacteria, especially Gram-negative bacteria, induce RegIIIγ expression on ECs, and a recent study demonstrated that MyD88 intrinsically expressed on ECs controls the production of RegIIIγ, which establishes the physical separation between the microbiota and the intestinal epithelial surface [21].

Unlike RegIIIγ, which specifically targets Gram-positive bacteria, bactericidal and/or permeability-increasing protein (BPI) shows antimicrobial activity against Gram-negative bacteria. The high affinity of BPI for lipopolysaccharide (LPS) leads to the

destabilization of the outer membrane of Gram-negative bacteria and also neutralizes LPS-induced inflammation [22].

Antimicrobial enzymes

Antimicrobial activity is also mediated by bacteriolysis enzymes (e.g. secretory phospholipase A2 and lysozyme). Phospholipase A2 is a small enzyme produced by Paneth cells that degrades bacterial phospholipids and subsequently disrupts the integrity of Gram-positive and -negative bacteria [23]. Phospholipase A2 enzyme activity is normal in the intestine of germ-free rats [24], but caloric restriction increases the gene expression of lysozyme and phospholipase A2 [25]. Therefore, it is likely that nutritional conditions rather than commensal bacteria regulate the activity of these antimicrobial enzymes in the intestine. Lysozyme is produced by Paneth cells and ECs. Its bactericidal activity derives from its cleavage of the glycosidic linkage between *N*-acetylglucosamine and *N*-acetyl muramic acid of peptidoglycan. Because Gram-positive bacteria express more peptidoglycan than Gram-negative bacteria, lysozyme acts preferentially on Gram-positive bacteria.

Transport of antibodies through ECs

IgA transport mediated by polymeric immunoglobulin receptors

One function of the epithelial immune barrier is to transport antibodies across the barrier. ECs express polymeric immunoglobulin receptors (pIgR) for the transport of polymeric forms of IgA (pIgA) and IgM (pIgM) in the basal-to-apical direction in association with an extracellular proteolytic fragment of the pIgR (known as the secretory component) [26]; together, the IgA and the secretory component form secretory immunoglobulin A (S-IgA). After S-IgA is secreted into the intestinal lumen, it inhibits adherence of pathogens to host ECs in the intestine and neutralizes pathogenic toxins by binding to their biologically active sites (Fig. 1) [27]. Additionally, IgA is able to exclude antigens and pathogens from the intestinal secretions while it is transported through ECs, and it also prevents viral replication inside ECs [28,29].

In addition to the function of S-IgA in the immunosurveillance, several lines of evidence demonstrate that S-IgA has a key role in preventing the penetration and/or growth of commensal bacteria [30]. These functions of S-IgA achieve the immune responses against commensal bacteria restricted in the intestinal but not systemic immune compartments in normal mice, while IgA-deficient mice exhibited systemic IgG responses against commensal bacteria [31–33]. A recent study also demonstrated that, in the absence of IgA, commensal bacteria-derived stimulation induced the increased expression of interferon-regulated genes in the ECs for the compensatory immunosurveillance with simultaneous reduction of lipid metabolism-related Gata4-regulated genes, which resulted in the lipid malabsorption and decreased lipid deposition [34]. Thus, S-IgA mediates the regulation between ECs and commensal bacteria, which is important not only for the maintenance of immunological homeostasis but also for metabolism [34].

Neonatal Fc receptor for IgG transport

Another receptor for immunoglobulin is the neonatal Fc receptor for IgG (FcRn). Although early studies in rodents indicated that FcRn was responsible for the passive acquisition of IgG neonatally, subsequent studies indicated that FcRn is also expressed by adult human epithelium and antigen-presenting cells in the intestine and thus is not strictly limited to neonatal life [35]. Unlike pIgR mentioned above, human FcRn binds IgG and the transport pathway is bidirectional, both apical to basal and basal to apical [36]. The bidirectional transport of IgG enables retrieval of intestinal antigens in a complex with IgG into the intestinal lamina propria, where the antigen and/or IgG complexes are subsequently taken up by antigen-presenting cells to prime T cell responses [37].

Intraepithelial T lymphocytes

The epithelium also includes lymphocytes that are commonly termed intraepithelial lymphocytes (IELs) [38]. IELs reside between the basolateral surfaces of ECs, and one IEL occurs for every 4–10 ECs in the small intestine and for every 30–50 ECs in the large intestine.

Most IELs are T cells. As similar to T cells observed at other sites (e.g. spleen and intestinal lamina propria), some portions of IELs express αβ T cell receptors and act as cytotoxic T lymphocytes by recognizing antigenic peptides presented by classical major histocompatibility complex (MHC) molecules on pathogenic ECs (e.g. microbe-infected cells) and killing them by producing cytotoxic molecules (e.g. perforin and granzymes) [38]. Other IELs express the γδ T cell receptor (and are therefore known as γδIELs) and show minimal pathogen-specific activity [38,39]. The innate immune function of γδIELs enables the rapid removal of infected ECs. To recognize the infected ECs, non-classical MHC molecules, such as MHC class I chain-related protein A/B (MICA/B) in human, act as ligands for γδIELs. MICA/B is generally not expressed on ECs, but is induced by stresses such as heat shock and microbial infections. The activated γδIELs then synthesize an array of cytokines, including interleukin (IL)-2, IL-3, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, and cytotoxic molecules, such as perforin, granzyme, and Fas ligand to kill the microbe-infected ECs [38].

Epithelium senses signals from commensal bacterial in the regulation of T cell differentiation in the intestine

The immune system requires interactions with commensal bacteria for its development. Toll-like receptors (TLRs) act as sensors of commensal bacteria although they were initially discovered as pathogen recognition receptors. ECs express several kinds of TLRs and the ligands from commensal bacteria promote immunological functions of Ecs, such as IgA transport, tight junctions, and expression of antimicrobial peptides [40]. Of note, ECs have unique expression profiles and spatially restricted distribution (apical vs. basolateral) of TLRs together with unique underlying signaling pathways, which enables the prevention of deleterious inflammatory responses in the intestine [40].

Because commensal bacteria express shared molecules which act as a ligand of TLRs, it was previously thought that unspecified commensal bacteria indiscriminately induced the development of the immune system; however, accumulating evidence has demonstrated that individual species of commensal bacteria have specific roles in the determination of immunological balance by regulating T cell differentiation in the intestine [8]. ECs have an important role in this pathway.

Segmented filamentous bacteria induce the differentiation of Th17 cells

Several groups have shown that segmented filamentous bacteria (SFB) induce components of the active immune system, including IgA-producing cells, γδIELs, and IL-17-producing T (Th17) cells [41–43]. SFB colonization on ECs results in the production of serum amyloid A, which acts on intestinal dendritic cells (DCs) to enhance the production of IL-6 and IL-23 [43]. Because these two cytokines are Th17 cell-inducing cytokines, the immunological environment mediated by SFB, ECs, and DCs results in the preferential induction of Th17 cells in the intestine.

Preferential induction of Treg cells in the colon by Clostridium *clusters IV and XIVa*

Another form of crosstalk between ECs and commensal bacteria in the regulation of T cell differentiation is mediated by *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *coccoides* groups) [44]. By contrast to the effects of SFB, colonization by *Clostridium* clusters IV and XIVa induces regulatory T (Treg) cells in the colon to achieve quiescent immunity. *Clostridium* clusters IV and XIVa form a thin colonizing layer on the epithelium, where they enhance the release of the active form of TGF-β by increasing the expression of matrix metalloproteinases that convert latent TGF-β into the active form. Because TGF-β is an essential cytokine for the differentiation of Treg cells from naive T cells, colonization with these *Clostridium* species converts non-Treg cells into Treg cells locally in the colon with little effect on thymus-derived Treg cells.

Dietary metabolites regulate intestinal immunity through the epithelium

Nutritional materials also influence intestinal immunity, and commensal bacteria are involved in metabolizing indigestible dietary materials into biologically active metabolites. Dietary materials (e.g. polysaccharides, vitamins, and lipids) and their metabolites contribute to the regulation of intestinal immunity (Fig. 2).

Polysaccharides

Dietary polysaccharides and endogenous mucus in the intestine are digested and metabolized into short-chain fatty acids, such as acetate, butyrate, and propanoate, by bacterial fermentation. These short-chain fatty acids are an energy source for ECs and affect immune cell functions. For example, acetate and butyrate maintain epithelial barrier function by stimulating the release of mucin and by facilitating the maintenance of epithelial integrity [45,46]. Acetate and butyrate also regulate the proliferation of ECs and their production of cytokines [47,48]. In addition, acetate modulates the immunological function of neutrophils that express G-protein-coupled receptor 43 [GPR43, also known as free fatty acid receptor 2 (FFAR2)], a receptor for the short-chain fatty acids. Neutrophils lacking GPR43 show decreased levels of phagocytic activity and lower production of reactive oxygen species, but also are more responsive to chemoattractants such as C5a and inflammatory chemokines [49]. Consistent with these findings, intestinal inflammation is exacerbated in GPR43-deficient mice.

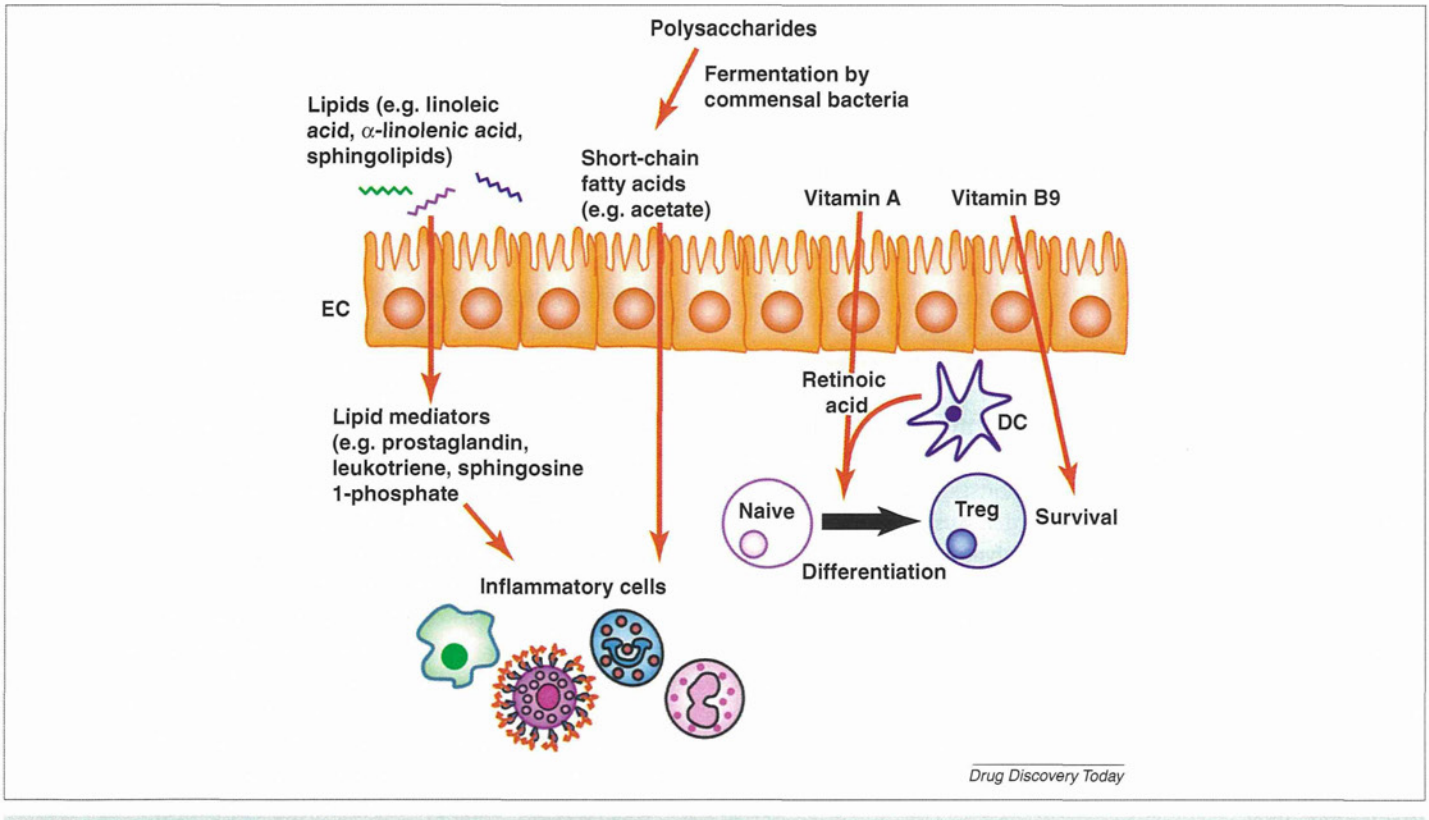

FIGURE 2

Dietary materials in the regulation of EC functions. Dietary lipids are metabolized into lipid mediators, and short-chain fatty acids are generated by fermentation of polysaccharides by commensal bacteria. These products positively or negatively regulate the functions of inflammatory cells. ECs also absorb vitamin A, and both ECs and dendritic cells (DCs) metabolize vitamin A into retinoic acid, which preferentially induces regulatory T (Treg) cells from naive T cells. The differentiated Treg cells require vitamin B9 for their survival. *Abbreviation:* EC, enterocytes.

Vitamins

Vitamins are supplied by both the diet and commensal bacteria. Several lines of evidence have shown that vitamins are involved in regulating immune responses through the epithelium. For example, retinoic acid, a metabolite of vitamin A, is involved in the preferential induction of regulatory T cells and the inhibition of Th17 cells [50]. Both ECs and DCs in the intestine are the major cell types that express retinaldehyde dehydrogenase, a key enzyme for the conversion of vitamin A into retinoic acid, suggesting that the unique gut environment mediated by ECs, DCs, and vitamin A preferentially induces Treg cells for maintaining quiescent immunity in the intestine. Because it was reported that Treg cells enhanced the differentiation of IgA^1 B cells in the intestine [51,52] and retinoic acid induced the expression of gut-homing molecules (e.g. CCR9 and $\alpha4\beta7$ integrin) on IgA-committed B cells as well as T cells [53,54], it is likely that retinoic acid directly and indirectly enhances intestinal IgA responses.

Vitamin B9 is another important vitamin in the maintenance of Treg cells. Vitamin B9 receptor (folate receptor 4) is exclusively expressed on Treg cells and can therefore be used as a cell surface marker of Treg cells [55]. We recently showed that vitamin B9 is an essential survival factor for Treg cells [56]. Indeed, Treg cells differentiate from naive T cells but fail to survive in vitamin B9-reduced conditions. Because vitamin B9 is supplied from both the diet and commensal bacteria, and dietary vitamin B9 is predominantly absorbed by ECs in the jejunum and duodenum, depletion of dietary vitamin B9 results in the reduction of Treg cells in the small intestine.

Lipids

Dietary lipids also involved in the regulation of intestinal immune responses. The ratio of omega-3 polyunsaturated fatty acids (ω-3 PUFA) to ω-6 PUFA in the diet may determine the presence and/or levels of inflammatory conditions. Dietary linoleic acid is the parent fatty acid of ω-6 PUFA which is metabolized into proinflammatory lipid mediators, whereas ω-3 PUFA, which is derived from dietary linolenic acid, is metabolized into anti-inflammatory mediators [57]. A possible molecular mechanism is that ω-3 PUFA exert anti-inflammatory effects through binding to GPR120, which is mostly expressed by macrophages, thereby inhibiting the production of inflammatory cytokines [58].

Another lipid metabolite with important immunological function is sphingosine 1-phosphate (S1P), which regulates cell trafficking, activation, and survival. Intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues and diet could be a major source of sphingolipids in the intestine, especially sphingomyelin from meat, milk, eggs, and fish [59]. Because ECs express alkaline sphingomyelinase and ceramidase to degrade dietary sphingomyelin into ceramide and sphingosine, respectively, and also express several key enzymes in the production of S1P from ceramide and sphingosine (e.g. sphingosine kinase), it is possible that ECs produce ceramide, sphingosine, and S1P for the regulation of intestinal immune responses.

Concluding remarks

ECs in the intestine have both physical and immunological barrier functions, which are achieved by immunological communication with both immunocompetent cells and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites). Elucidation of the complex networks established by commensal bacteria, dietary molecules, and the host immune system will provide new insights in gut environment-based mucosal immunology and should lead to new strategies to prevent and treat infectious and immune diseases in the intestine.

Acknowledgments

The work related to this review was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Young Scientists A [22689015 to J.K.], for Challenging Exploratory Research [24659217 to J.K.], for Scientific Research on Innovative Areas [23116506 to J.K.], for Scientific Research S [23229004 to H.K.], and for Scientific Research on Priority Area [19059003 to H.K.], Leading-edge Research Infrastructure Program [J.K. and H.K.]); the Ministry of Health and Welfare of Japan (to J.K. and H.K.); the Global Center of Excellence Program of the Center of Education and Research for Advanced Genome-based Medicine (to H.K.); the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (to J.K.); and the Yakult Bio-Science Foundation (to J.K.).

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A Pivotal Role of Vitamin B9 in the Maintenance of Regulatory T Cells *In Vitro* and *In Vivo*

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Abstract

Dietary factors regulate immunological function, but the underlying mechanisms remain elusive. Here we show that vitamin B9 is a survival factor for regulatory T (Treg) cells expressing high levels of vitamin B9 receptor (folate receptor 4). In vitamin B9-reduced condition *in vitro*, Treg cells could be differentiated from naïve T cells but failed to survive. The impaired survival of Treg cells was associated with decreased expression of anti-apoptotic Bcl2 and independent of IL-2. *In vivo* depletion of dietary vitamin B9 resulted in the reduction of Treg cells in the small intestine, a site for the absorption of dietary vitamin B9. These findings provide a new link between diet and the immune system, which could maintain the immunological homeostasis in the intestine.

Citation: Kunisawa J, Hashimoto E, Ishikawa I, Kiyono H (2012) A Pivotal Role of Vitamin B9 in the Maintenance of Regulatory T Cells *In Vitro* and *In Vivo*. PLoS ONE 7(2): e32094. doi:10.1371/journal.pone.0032094

Editor: Jacques Zimmer, Centre de Recherche Public de la Santé (CRP-Santé), Luxembourg

Received November 16, 2011; **Accepted** January 18, 2012; **Published** February 20, 2012

Funding: This work was supported by grants from: the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Young Scientists A [22689015 to J.K.] for Scientific Research on Innovative Areas [23116506 to J.K.], for Scientific Research S [23229004 to H.K.], for Scientific Research on Priority Area [19059003 to H.K.], and for JSPS Fellows (021-07124 to Y.K.); the Ministry of Health and Welfare of Japan (to J.K. and H.K.); the Global Center of Excellence Program of the Center of Education and Research for Advanced Genome-based Medicine (to H.K.); the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN; to J.K.); and the Yakult Bio-Science Foundation (to J.K.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

To achieve immunosurveillance and immunological homeostasis at the interface between the interior and exterior of the gastrointestinal tract, the intestinal immune system tightly balances states of immune activation and quiescence [1]. Thus, gastrointestinal tissues contain numerous kinds of T cells, such as Th1, Th2, Th17, forkhead box P3 (Foxp3)$^+$ regulatory T (Treg) cells, IL-10–producing Foxp3$^-$ T regulatory type 1 cells, and T cells expressing γδ T cell receptor, which together create the appropriate immunological environment.

Th17 and Treg cells are observed most frequently in the intestine, and their preferential differentiation is achieved by a unique cytokine environment created by transforming growth factor β (TGF-β), IL-6, and IL-23 [2]. In addition to these host-derived factors, the development and function of the immune system are influenced by crosstalk with environmental factors [3]. For example, stimulation by segmented filamentous bacteria results in the preferential induction of Th17 cells, whereas colonic Treg cells are induced by crosstalk between epithelial cells and Clostridium clusters IV and XIVa [4,5,6].

Nutritional molecules are also considered to be essential environmental factors for the development, maintenance, and regulation of gut immune responses. Thus, deficient or inappropriate nutritional intake increases the risk of infectious, allergic, and inflammatory diseases [7,8]. Among various dietary factors, vitamins are important participants in the regulation of immune responses. For example, vitamin A is converted into retinoic acid (RA) by gut-associated dendritic cells; RA induces the expression of gut-homing molecules (e.g., α4β7 integrin and CCR9) on activated T and B cells [9,10] and promotes the preferential differentiation of Treg cells and the simultaneous inhibition of Th17 cells [11,12,13,14]. Vitamin B6 is required for the metabolic pathway of sphingosine 1-phosphate, a lipid mediator that regulates cell trafficking [15]; disruption of vitamin B6 function results in aberrant T-cell differentiation and cell trafficking in both systemic and intestinal compartments [16,17,18].

Vitamin B9 (also known as folate and folic acid) is a water-soluble vitamin derived from both diet and commensal bacteria [19]. Vitamin B9 is essential for the synthesis, replication, and repair of nucleotides for DNA and RNA and is thus required for cell proliferation and survival [20]. Methotrexate (MTX) acts as a vitamin B9 antagonist and blocks vitamin B9–mediated nucleotide synthesis, making MTX useful as an anti-tumor [21] and anti-rheumatoid arthritis agent [22]. Vitamin B9 deficiency also reduces the proliferative responses of lymphocytes and natural killer cell activity [23,24]. Additionally, the vitamin B9 receptor folate receptor 4 (FR4) is both a marker of Treg cells and is immunologically functional [25]; however, how it functions in the intestinal immune system is largely unknown. In this study, we examined the role of vitamin B9 in the regulation of Treg cell *in vitro* and *in vivo*.

Materials and Methods

Mice and experimental treatment

Female Balb/c mice (7–9 wk of age) were purchased from Japan Clea (Tokyo, Japan). Vitamin B9–deficient and control

diets composed of chemically defined materials (Oriental Yeast, Tokyo, Japan) were used within 3 months. All animals were maintained in the experimental animal facility at the University of Tokyo, and the experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines (Approval #20–28).

Lymphocyte isolation

Lymphocytes were isolated from the lamina propria (LP), as previously described [18,26]. Briefly, lymphocytes were isolated from dissected PPs by enzymatic dissociation using collagenase (Wako, Osaka, Japan). To isolate lymphocytes from the LP of jejunum/duodenum, PPs were removed and the remaining intestinal tissue was cut into 2-cm pieces and stirred in RPMI 1640 medium containing 1 mM EDTA and 2% fetal calf serum (FCS). The tissue pieces were then stirred in 0.5 (for small intestine) or 1.0 (for large intestine) mg/mL collagenase, and the dissociated cells were subjected to centrifugation through a discontinuous Percoll gradient. Lymphocytes were isolated at the interface between the 40% and 75% Percoll layers.

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as previously described [18,26]. Cells were pre-incubated with anti-CD16/32 antibodies and then stained with fluorescent antibodies specific for CD4, ICOS, and GITR (BD Biosciences, San Jose, CA) and FR4 (Biolegend). A Via-probe solution (BD Biosciences) was used to discriminate between dead and living cells. Intracellular staining of Foxp3 (eBioscience, San Diego, CA), phosphorylated STAT5, Ki67 and Bcl2 (BD Biosciences) was performed in accordance with the manufacturers' instructions. Flow cytometry and cell sorting were carried out using the FACSCantoII and FACSAria systems (BD Biosciences), respectively.

Vitamin B9 measurement

To measure vitamin B9 concentrations, intestinal washes were collected by washing 12 cm of jejunum/duodenum or whole colon with 1 mL of PBS. The vitamin B9 concentration in intestinal washes and serum was measured with a RIDASCREEN enzyme immunoassay kit (R-Biopharm AG, Darmstadt, Germany) in accordance with the manufacturer's instructions. To measure the amounts of intracellular vitamin B9, 5×10^6 purified cells were washed twice with PBS, and a cell lysate was obtained by homogenizing cells in PBS containing 0.01% NP-40. After cell debris was removed by centrifugation, vitamin B9 amounts in the supernatant were measured with a RIDASCREEN enzyme immunoassay kit.

In vitro culture

For the induction of Treg cells from naïve T cells, $CD62L^{hi}CD4^+$ naïve T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody and 1 μg/mL of an anti-CD28 antibody (BD Biosciences) plus 2 ng/mL of human TGF-β (PeproTech, Rocky Hill, NJ) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS. To examine the maintenance of differentiated Treg cells, purified $CD25^+CD4^+$ T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody with or without 1000 units/mL of IL-2 (Peprotech) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS in the presence or absence of 100 nM MTX.

Statistics

Results were compared with the Student's *t*-test by using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was established at $P<0.05$.

Results

Vitamin B9 is required for the survival of $Foxp3^+$ Treg cells

$Foxp3^+$ Treg cells express high levels of FR4, which is essential for their maintenance [25]. We therefore examined whether vitamin B9 is required for the differentiation of Treg cells from naïve T cells, the survival of differentiated Treg cells, or both. To address this, we initially performed an *in vitro* T-cell differentiation assay. Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in complete or vitamin B9–reduced medium. Although a small amount of vitamin B9 is supplied from fetal calf serum (FCS) even in vitamin B9–null medium (0.2 ppb, compared with 25 ppb in normal medium), the total cell number was decreased in the condition with reduced vitamin B9 compared to the control; however, $Foxp3^+$ Treg cells were generated at a normal frequency (Fig. 1A).

To investigate the effects of vitamin B9 on differentiated Treg cells, we cultured $CD25^+$ Treg cells with anti-CD3 antibodies. The total cell number was significantly lower in the vitamin B9–reduced condition than in the control condition (Fig. 1B). The reduction in cell number occurred predominantly among the $Foxp3^+CD4^+$ Treg cells (Fig. 1B). The reduction of $FR4^{hi}Foxp3^+$ T cells was dependent on the dose of vitamin B9 (Fig. 1C).

We then measured the expression of Ki67 and anti-apoptotic Bcl-2 to investigate whether decreased number of $Foxp3^+CD4^+$ Treg cells in vitamin B9–reduced medium was due to the defects of cell proliferation, survival, or both. We found that both Ki67 and Bcl2 were decreased in $Foxp3^+CD4^+$ Treg cells cultured in vitamin B9 vitamin B9–reduced medium, but magnitude of Bcl2 reduction was higher than Ki67 reduction (Fig. 2A and B). These findings suggest that vitamin B9 is preferentially but not exclusively required for the survival of Treg cells *in vitro*.

Vitamin B9 carrier-mediated pathway is not specifically involved in the survival of Treg cells

Because vitamin B9 is highly hydrophilic, mammalian cells must actively mediate the entry of vitamin B9 into cells by carrier- or receptor-mediated pathways [27]. Carriers include the proton-coupled folate transporter and the reduced folate carrier [27]. To examine whether a carrier-mediated pathway is involved in maintaining Treg cells, we employed MTX, an antagonist of vitamin B9 that is transported mainly via the reduced folate carrier and rarely via folate receptors [28,29]. MTX treatment reduced the numbers of both Treg and non-Treg cells (Fig. 3), suggesting that the carrier-mediated pathway does not specifically maintain Treg cells.

Vitamin B9 is an IL-2–independent survival factor for Treg cells

Treg cells could vigorously proliferate in some circumstances (e.g., antigen-specific activation through their highly sensitive TCR signaling [30] and IL-2-mediated activation [31]), which led to a hypothesis that Treg cells simply require large amounts of vitamin B9 as a source of nucleotides, and thus Treg cells might express FR4 as an additional means of acquiring vitamin B9. If so, $FR4^{hi}$ Treg cells should contain a larger amount of vitamin B9 in the intracellular compartments; however, the amount of intracel-

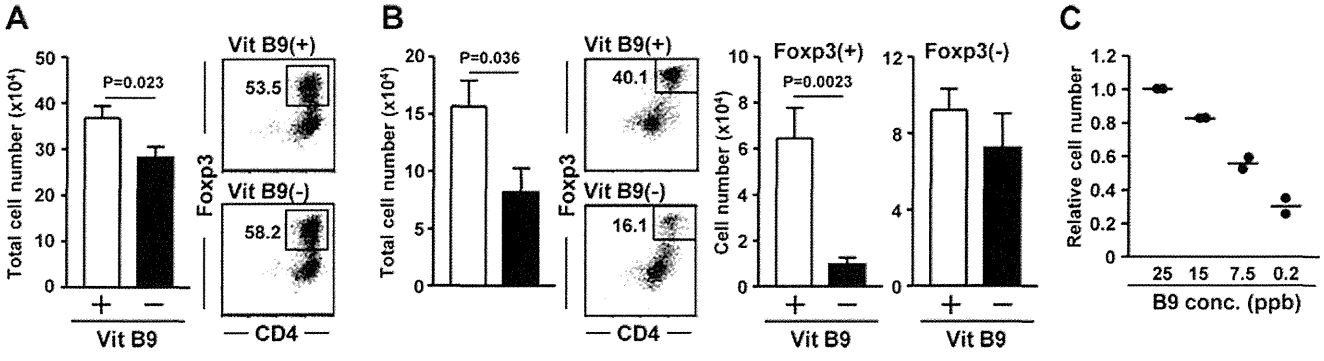

Figure 1. Requirement of vitamin B9 for the maintenance of Treg cells. (A) Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in the presence of normal [Vit B9(+)] or reduced [Vit B9(−)] amounts of vitamin B9. After 4 days, total cell numbers were calculated, and the differentiation into $Foxp3^+$ Treg cells was examined by flow cytometry. Data are means ± SEM (n = 4). (B) $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Cont or B9(−) medium. The frequencies of $Foxp3^+$ and $Foxp3^-CD4^+$ T cells (B) were determined by flow cytometry. Cell numbers were calculated using the total cell number and flow cytometric data. Data are means ± SEM (n = 6). (C) Experiments similar to that shown in (B) were performed with different concentrations of vitamin B9. The relative cell number of $Foxp3^+$ Treg cells is expressed as a ratio to the cell number in control medium. The values and means are indicated with dots and lines, respectively. Similar results were obtained from 2 independent experiments.
doi:10.1371/journal.pone.0032094.g001

lular vitamin B9 was equivalent between $FR4^{hi}$ Treg and $FR4^{low/-}$ non-Treg cells (Fig. 4A). Thus, FR4 might have an additional specific function for the survival of Treg cells.

IL-2 stimulation enhance the survival of Treg cells [31,32,33]. The FR4-mediated vitamin B9 signal might undergo crosstalk with IL-2-mediated signaling to maintain the survival of $FR4^{hi}Foxp3^+$ Treg cells. To test this, Treg cells were cultured with an anti-CD3 antibody together with IL-2. Although the absolute cell numbers were low in the reduced vitamin B9 condition, the magnitude of the IL-2-mediated enhancement of Treg cell growth was similar in the control and vitamin B9-reduced conditions (Fig. 4B). Consistent with this finding, comparable expression of phosphorylated STAT5 was noted in the control and vitamin B9-reduced conditions (Fig. 4C).

Dietary vitamin B9 maintains $Foxp3^+$ Treg cells in the small intestine

To examine whether vitamin B9 affects Treg cells *in vivo*, we maintained mice on a vitamin B9–depleted diet for 8 wk. Mice maintained with vitamin B9(−) diet showed less vitamin B9 in the small-intestinal wash than controls (Fig. 5A). In contrast, the amounts of vitamin B9 in the large-intestinal wash and serum were not different in those mice (Fig. 5A), presumably due to vitamin B9 production from commensal bacteria [19].

We then focused on Treg cells in the mice maintained with vitamin B9(−) diet. Consistent with our *in vitro* data, the small intestines of mice maintained with vitamin B9(−) diet had fewer $Foxp3^+$ Treg cells than those of control mice (p = 0.018), and there was no statistical difference (p = 0.3022) in the number of $Foxp3^-CD4^+$ non-Treg cells (Fig. 5B). The number of Treg and

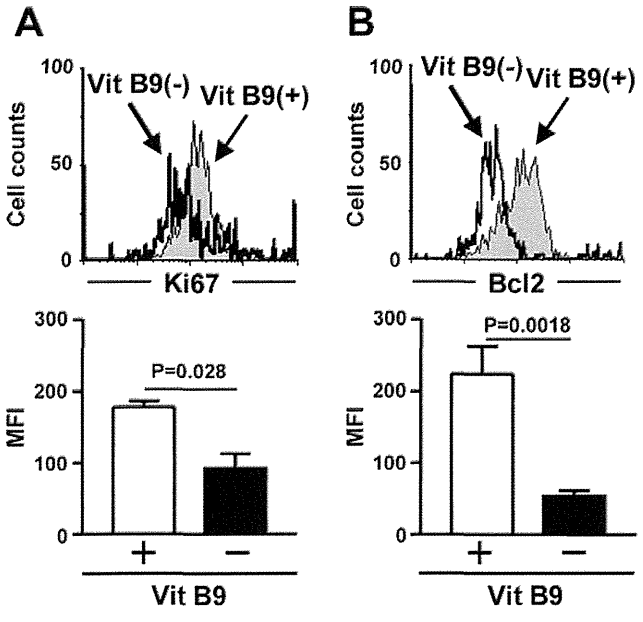

Figure 2. Vitamin B9 is essential for the survival of Treg cells. $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Vit B9(+) or Vit B9(−) medium. The expression of Ki67 (A) and Bcl2 (B) in $Foxp3^+CD4^+$ T cells were determined by flow cytometry (top panels) and graphs show the means fluorescent intensity (MFI; bottom panels). Data are means ± SD (n = 3). Data are representative of 4 independent experiments.
doi:10.1371/journal.pone.0032094.g002

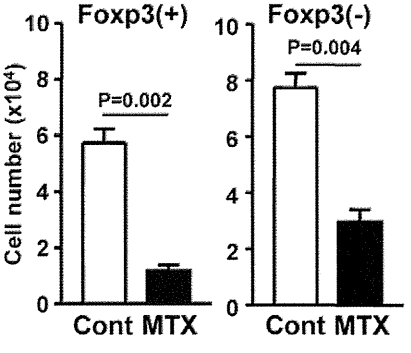

Figure 3. Vitamin B9 carrier-mediated pathway is not specific pathway in the maintenance of T cell survival. $CD25^+$ $CD4^+$ T cells were cultured with an anti-CD3 antibody in complete medium containing 100 nM methotrexate (MTX), and the frequency and absolute cell numbers of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells were determined. Data are means ± SEM (n = 4). Data are representative of two independent experiments.
doi:10.1371/journal.pone.0032094.g003

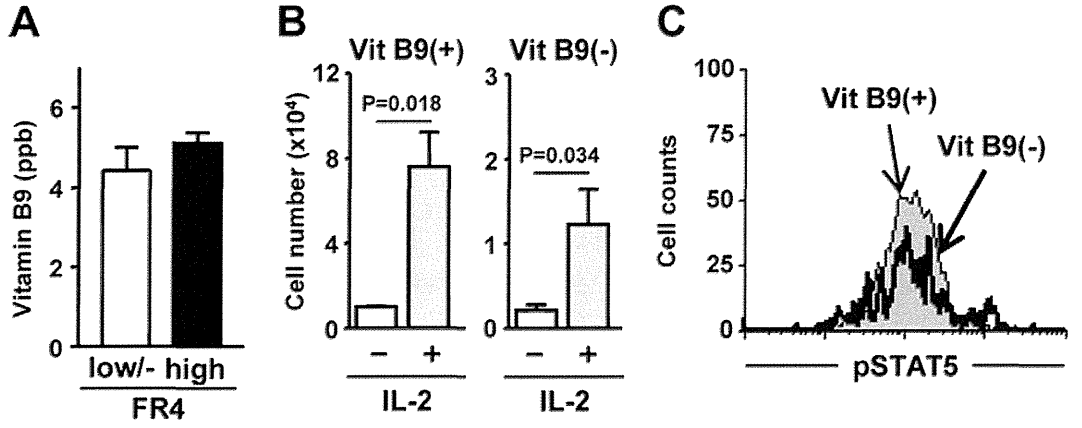

Figure 4. Vitamin B9 is IL-2-independent survival factor for Treg cells. (A) The amounts of intracellular vitamin B9 were measured using purified $CD4^+FR4^{hi}$ Treg or $CD4^+FR4^{low/-}$ non-Treg cells. Data are means ± SEM (n = 4). (B, C) Experiments similar to those shown in Fig. 1B were performed in the presence of anti-CD3 antibody stimulation with or without IL-2 stimulation. Cell number of $Foxp3^+CD4^+$ T cells (B) and the expression of phosphorylated STAT5 (pSTAT5) in $Foxp3^+CD4^+$ T cells (C) were determined. Data in (B) are means ± SEM (n = 6). Similar results were obtained from 3 separate experiments.
doi:10.1371/journal.pone.0032094.g004

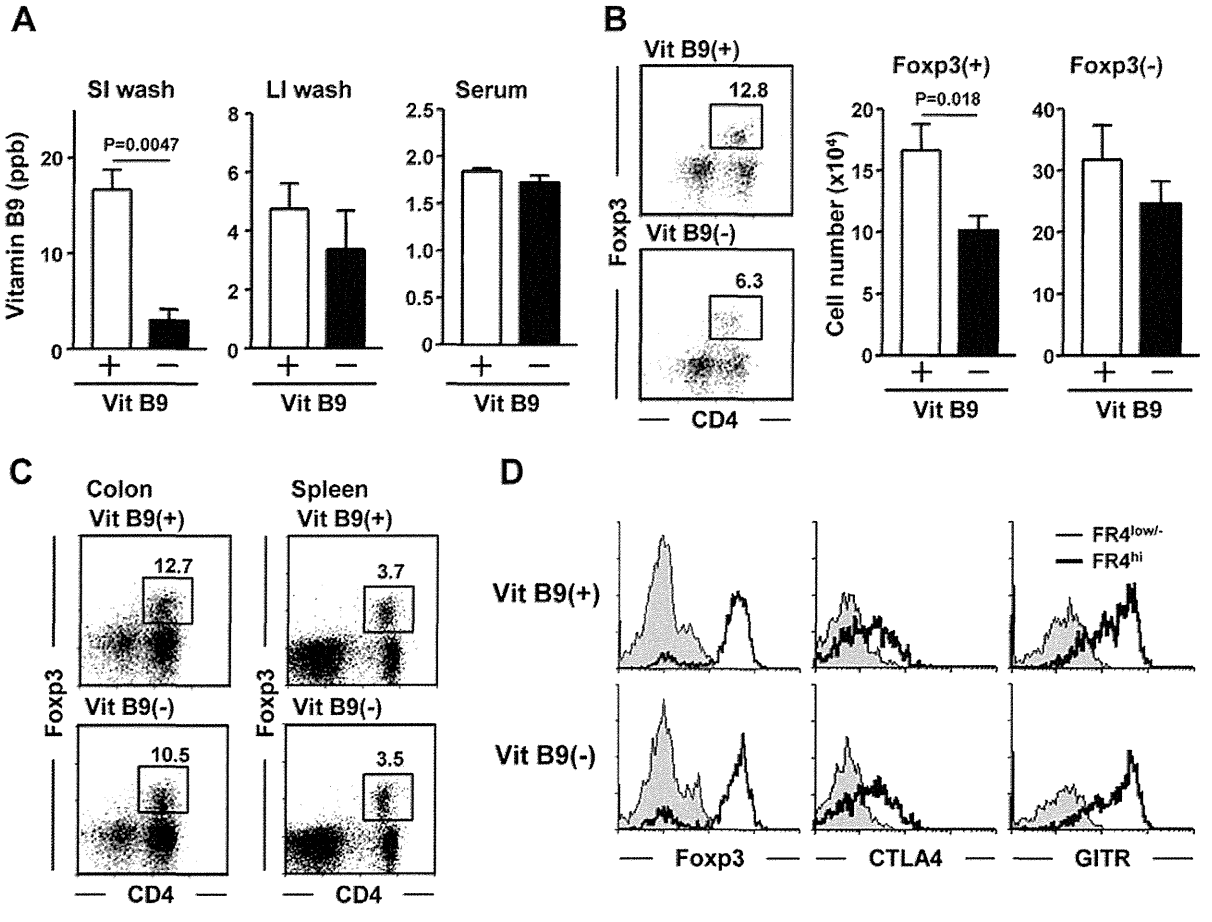

Figure 5. Depletion of dietary vitamin B9 selectively reduces Treg cells in the small intestine. Mice were maintained on a control [Vit B9(+)] or vitamin B9-depleted [Vit B9(−)] diet for 8 wk. (A) Vitamin B9 concentrations were measured in intestinal washes of the small intestine (SI), large intestine (LI), and serum. The data are mean ± SEM (n = 6). (B, C) The frequency and cell numbers of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells in the small intestine (B), colon, and spleen (C) were calculated using the total cell number and flow cytometric data (mean ± SEM, n = 6). (D) Flow cytometric analysis was performed to determine the expression levels of Foxp3, CTLA4, and GITR on the surface of $FR4^{low/-}$ (thin line) and $FR4^{hi}$ (thick line) $CD4^+$ T cells in the LP. Similar results were obtained from 3 separate experiments.
doi:10.1371/journal.pone.0032094.g005