pose a conflict of interest.

Abbreviations : Ab, antibody; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HSV, herpes simplex virus; IHL, intrahepatic lymphocyte; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; JEV, Japanese encephalitis virus; Lin, lineage; mDC, myeloid DC; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; Poly IC, polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene-I; SNPs, single nucleotide polymorphisms; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β

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ABSTRACT

The polymorphisms in *IL-28B* (IFN-λ3) gene are strongly associated with the efficacy of HCV clearance. Dendritic cells (DCs) sense HCV and produce IFNs, thereby playing some cooperative roles with HCV-infected hepatocytes in the induction of interferon-stimulated genes (ISGs). BDCA3^{+} DCs are discovered as a producer of IFN-λ upon toll-like receptor 3 (TLR3) agonist. We thus aimed to clarify the roles of BDCA3^{+}DCs in anti-HCV innate immunity. Seventy healthy subjects and 20 patients with liver tumors were enrolled. BDCA3^{+}DCs, in comparison with plasmacytoid DCs and myeloid DCs, were stimulated with TLR agonists, cell-cultured HCV (HCVcc) or Huh7.5.1 cells transfected with HCV/JFH-1. BDCA3^{+}DCs were treated with anti-CD81 antibody, inhibitors for endosome acidification, TRIF-specific inhibitor or ultraviolet-irradiated HCVcc. The amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2 and IL-28B were quantified by subtype-specific ELISA. The frequency of BDCA3^{+}DCs in PBMC was extremely low but higher in the liver. BDCA3^{+}DCs recovered from PBMC or the liver released large amounts of IFN-λs, when stimulated with HCVcc or HCV-transfected Huh7.5.1.

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BDCA3$^+$DCs were able to induce ISGs in the co-existing JFH-1-positive Huh7.5.1 cells. The treatments of BDCA3$^+$DCs with anti-CD81 antibody, cloroquine or bafilomycin A1 reduced HCVcc-induced IL-28B release, whereas BDCA3$^+$DCs comparably produced IL-28B upon replication-defective HCVcc. The TRIF-specific inhibitor reduced IL-28B release from HCVcc-stimulated BDCA3$^+$DCs. In response to HCVcc or JFH-1-Huh7.5.1, BDCA3$^+$DCs in healthy subjects with IL-28B major (rs8099917, TT) released more IL-28B than those with IL-28B minor genotype (TG). ***Conclusion:*** Human BDCA3$^+$DCs, having tendency of being accumulated in the liver, recognize HCV by a CD81-, endosome- and TRIF-dependent manner and produce substantial amounts of IL-28B/IFN-λ3, the ability of which is superior in subjects with IL-28B major genotype.

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most serious health problems in the world. More than 170 million people are chronically infected with HCV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma. Genome-wide association studies have successfully identified the genetic polymorphisms (single nucleotide polymorphisms, SNPs) upstream of the promoter region of the IL-28B/IFN-λ3 gene which are strongly associated with the efficacy of pegylated interferon-α (PEG-IFN-α) and ribavirin therapy or spontaneous HCV clearance (1-4).

IFN-λs, or types III IFNs, comprise a family of highly homologous molecules consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). In clear contrast with type I IFNs, they are released from relatively restricted types of cells, such as hepatocytes, intestinal epithelial cells or dendritic cells (DCs). Also, the cells that express hetero-dimeric IFN-λ receptors (IFN-λR1 and IL-10R2) are restricted to cells of epithelial origin, hepatocytes or DCs (5). Such limited profiles of cells expressing IFN-λs and their receptors define the biological uniqueness of IFN-λs. It has been shown that IFN-λs convey anti-HCV activity by inducing

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various interferon-stimulated gens (ISGs) (5), the profiles of which were overlapped but others were distinct from those induced by IFN-α/β. Some investigators showed that the expression of IL-28 in PBMC was higher in subjects with IL-28B major than those with minor, however the levels of IL-28 transcripts in liver tissue were comparable regardless of IL-28B genotype (2, 6).

At the primary exposure to hosts, HCV maintains high replicative levels in the infected liver, resulting in the induction of IFNs and ISGs. In a case of successful HCV eradication, it is postulated that IFN-α/β and IFN-λ cooperatively induce anti-viral ISGs in HCV-infected hepatocytes. It is of particular interest that, in primary human hepatocytes or chimpanzee liver, IFN-λs, but not type-I IFNs, is primarily induced after HCV inoculation, the degree of which is closely correlated with the levels of ISGs (7). These results suggest that hepatic IFN-λ could be a principal driver of ISG induction in response to HCV infection. Nevertheless, the possibility remains that DCs, as a prominent IFN producer in the liver, play significant roles in inducing hepatic ISGs and thereby suppressing HCV replication.

Dendritic cells (DCs), as immune sentinels, sense specific genomic and/or structural components of pathogens with various pattern recognition receptors and eventually release IFNs and inflammatory cytokines (8). In general, DCs migrate to the organ where inflammation or

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cellular apoptosis occurs and alter their function in order to alleviate or exacerbate the disease conditions. Therefore, the phenotypes and/or capacity of liver DCs are deemed to be influenced in the inflamed liver. In humans, the existence of phenotypically and functionally distinct DC subsets has been reported: myeloid DC (mDC) and plasmacytoid DC (pDC) (9). Myeloid DCs predominantly produce IL-12 or TNF-α following pro-inflammatory stimuli, while pDCs release considerable amounts of type I IFNs upon virus infection (9). The other type of mDCs, mDC2 or BDCA3$^+$(CD141) DCs, have been drawing much attention recently, since human BDCA3$^+$DCs are reported to be a counterpart of murine CD8α$^+$DCs (10). Of particular interest is the report that BDCA3$^+$DCs have a potent capacity of releasing IFN-λ in response to toll-like receptor 3 (TLR3) agonist (11). However, it is still largely unknown whether human BDCA3$^+$DCs are able to respond to HCV.

Taking these reports into consideration, we hypothesized that human BDCA3$^+$DCs, as a producer of IFN-λs, have crucial roles in anti-HCV innate immunity. We thus tried to clarify the potential of BDCA3$^+$DCs in producing type III IFNs, by using cell-cultured HCV (HCVcc) or hepatoma cells harboring HCV as stimuli. Our findings show that BDCA3$^+$DCs are quite unique DC subset, characterized by a potent and specialized ability to secrete IFN-λs in

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response to HCV. The ability of BDCA3^{+}DCs to release IL-28B upon HCV is superior in subjects with IL-28B major (rs8099917, TT) to those with minor (TG or GG) genotype, suggesting that BDCA3^{+}DCs are one of the key players in IFN-λ-mediated innate immunity.

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MATERIALS AND METHODS

Subjects.

This study enrolled 70 healthy volunteers (male / female; 61 / 9) (age; mean ± SD, 37.3 ± 7.8 yrs.) and 20 patients who underwent surgical resection of liver tumors at Osaka University Hospital (**Supplementary table 1).** The study was approved by the ethical committee of Osaka University Graduate School of Medicine. Written informed consent was obtained from all of them. All healthy volunteers are negative for HCV, hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and had no apparent history of liver, autoimmune or malignant diseases.

Reagents.

The specifications of all antibodies used for FACS or cell sorting TLR-specific synthetic agonists, pharmacological reagents and inhibitory peptides are listed in the supplementary materials.

Separation of dendritic cells from PBMC or intra-hepatic lymphocytes.

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We collected 400 mL of blood from each healthy volunteer and processed for PBMCs. Non-cancerous liver tissues were obtained from patients who underwent resection of liver tumors (**Supplementary table**). For the collection of intrahepatic lymphocytes (IHLs), liver tissues were washed thoroughly with phosphate-buffered saline to remove the peripheral blood adhering to the tissue and ground gently. After Lin-negative ($CD3^-$, $CD14^-$, $CD19^-$ and $CD56^-$) cells were obtained by the MACS system, each DC subset with the defined phenotype was sorted separately under FACS Aria (BD). The purity was more than 98%, as assessed by FACS Canto II (BD). Sorted DCs were cultured at $2.5x10^4$/well on 96-well culture plates.

Immunofluorescence staining of human liver tissue

Tissue specimens were obtained from surgical resections of non-cancerous liver from the patients as described above. Briefly, the 5μm sections were incubated with the following antibodies: mouse biotinylated anti-human BDCA3 antibody (Miltenyi-Biotec), and mouse anti-human CLEC9A antibody (Biolegend) and subsequently with secondary goat anti-rabbit Alexa Fluor®488 or goat anti-mouse Alexa Fluor®594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-GTM (SouthernBiotech,

Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Cells and viruses.

The *in vitro* transcribed RNA of the JFH-1 strain of HCV was introduced into FT3-7 cells (12) or Huh7.5.1 cells. The stocks of cell-cultured HCV (HCVcc) were generated by concentration of the medium from JFH-1-infected FT3-7 cells. The virus titers were determined by focus forming assay (13). The control medium was generated by concentration of the medium from HCV-uninfected FT3-7 cells. Infectious JEVs were generated from the expression plasmid (pMWJEATG1) as reported previously (14). HSV (KOS) was a generous gift from Dr. K. Ueda (Osaka University). Huh7.5.1 cells transduced with HCV JFH-1 strain was used for the co-culture with DCs. The transcripts of interferon-stimulated genes (ISGs) in Huh7.5.1 were examined by RT-PCR methods using gene-specific primers and probes (Applied Biosystems, Foster City, CA).

Secretion assays.

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IL-28B/IFN-λ3 was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system (15). IL-29/IFN-λ1, IL-28A/IFN-λ2 and IFN-β were assayed by commercially available ELISA kits (eBioscience, R&D and PBL, respectively). IFN-α was measured by cytometric beads array kits (BD) according to the manufacturer's instructions.

Statistical analysis.

The differences between two groups were assessed by the Mann-Whitney non-parametric U test. Multiple comparisons between more than two groups were analyzed by the Kruskal-Wallis non-parametric test. Paired-t tests were used to compare differences in paired samples. All the analyses were performed using GraphPad Prism software (San Diego, CA).

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RESULTS

Human BDCA3^{+}DCs are phenotypically distinct from pDCs and mDCs.

We defined BDCA3^{+}DCs as Lin^{-}HLA-DR^{+}BDCA3^{high+} cells (**Fig 1A, left, middle**), and pDCs and mDCs by the patterns of CD11c and CD123 expressions (**Fig 1A, right**). The level of CD86 on pDCs or mDCs is comparatively higher than those on BDCA3^{+}DCs (**Fig 1B**). The expression of CD81 is higher on BDCA3^{+}DCs than on pDCs and mDCs (**Fig 1B, Fig S1**). CLEC9A, a member of C-type lectin, is expressed specifically on BDCA3^{+}DCs as reported elsewhere (16), but not on pDCs and mDCs (**Fig 1B**).

Liver BDCA3^{+}DCs are more mature than the counterparts in the periphery.

BDCA3^{+}DCs in infiltrated hepatic lymphocytes (IHLs) are all positive for CLEC9A, but liver pDCs or mDCs are not (data not shown). The levels of CD40, CD80, CD83 and CD86 on liver BDCA3^{+}DCs are higher than those on the peripheral counterparts, suggesting that BDCA3^{+}DCs are more mature in the liver compared to those in the periphery (**Fig 1C**).

In order to confirm that BDCA3^{+}DCs are localized in the liver, we stained the cells

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with immunofluorescence Abs in non-cancerous liver tissues. Liver BDCA3^{+}DCs were defined as BDCA3^{+}CLEC9A^{+} cells **(Fig 1D)**. Most of the cells were found near vascular compartment or in sinusoid or the space of Disse of the liver tissue.

BDCA3^{+}DCs are scarce in PBMCs but more abundant in the liver.

The percentages of BDCA3^{+}DCs in PBMCs were much lower than those of the other DC subsets (BDCA3^{+}DCs, pDCs and mDCs, mean ± SD [%], 0.054 ± 0.044, 0.27 ± 0.21 and 1.30 ± 0.65) **(Fig 2A)**. The percentages of BDCA3^{+}DCs in IHLs were lower than those of the others (BDCA3^{+}DCs, pDCs and mDCs, mean ± SD [%], 0.29 ± 0.25, 0.65 ± 0.69 and 1.2 ± 0.94) **(Fig 2B)**. The percentages of BDCA3^{+}DCs in the IHLs were significantly higher than those in PBMCs from relevant donors **(Fig 2C)**. Such relative abundance of BDCA3^{+}DCs in the liver over that in the periphery was observed regardless of the etiology of the liver disease (**Supplementary Table 1**).

BDCA3^{+}DCs produce a large amount of IFN-λs upon poly IC stimulation.

We compared DC subsets for their abilities to produce IL-29/IFN-λ1, IL-28A/IFN-λ2,

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IL-28B/IFN-λ3, IFN-β, and IFN-α in response to TLR agonists. Approximately 4.0×10^4 of BDCA3$^+$DCs were recoverable from 400ml of donated blood from healthy volunteers. We fixed the number of DCs at 2.5×10^4 cells/100 μl for comparison in the following experiments.

BDCA3$^+$DCs have been reported to express mRNA for TLR1, 2, 3, 6, 8, and 10 (17). First, we quantified IL-28B/IFN-λ3 as a representative for IFN-λs after stimulation of BDCA3$^+$DCs with relevant TLR agonists. We confirmed that BDCA3$^+$DCs released IL-28B robustly in response to TLR3 agonist/poly IC but not to other TLR agonists **(Fig S2)**. In contrast, pDCs produced IL-28B in response to TLR9 agonist/CpG but much lesser to other agonists **(Fig S2)**. Next, we compared the capabilities of DCs inducing IFN-λs and IFN-β genes in response to relevant TLR agonists. BDCA3$^+$DCs expressed extremely high levels of IL-29, IL-28A and IL-28B transcripts compared to other DCs, whereas pDCs induced a higher level of IFN-β than other DCs **(Fig S3A)**.

Similar results were obtained with the protein levels of IFN-λs, IFN-β and IFN-α released from DC subsets stimulated with TLR agonists. BDCA3$^+$DCs produce significantly higher levels of IL-29, IL-28B, and IL-28A than the other DC subsets. In clear contrast, pDCs release a significantly larger amount of IFN-β and IFN-α than BDCA3$^+$DCs or

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mDCs **(Fig 3A, Fig S3B)**. As for the relationship among the quantity of IFN-λ subtypes from poly IC-stimulated BDCA3$^+$DCs, the levels of IL-29/IFN-λ1 and /IL-28B/IFN-λ3 were positively correlated ($R2=0.76$, $p<0.05$), and those of IL-28A/IFN-λ2 and IL-28B/IFN-λ3 were positively correlated as well ($R2=0.84$, $p<0.0005$), respectively (**Fig S3C**). These results show that the transcription and translation machineries of IFN-λs may be overlapped among IFN-λ subtypes in BDCA3$^+$DCs upon poly IC stimulation.

Liver BDCA3$^+$DCs sorted from IHLs possess ability to produce IL-28B in response to poly IC **(Fig 3B),** showing that they are comparably functional.

In response to poly IC, BDCA3$^+$DCs were capable of producing inflammatory cytokines as well, such as TNF-α, IL-6 and IL-12p70 (**Fig S4A**). By using Huh7 cells harboring HCV subgenomic replicons (HCV-N, genotype 1b), we confirmed that the supernatants from poly IC-stimulated BDCA3$^+$DCs suppressed HCV replication in an IL-28B concentration dependent manner (**Fig S4B**). Therefore, poly IC-stimulated BDCA3$^+$DCs are capable of producing biologically active substances suppressing HCV replication, some part of which may be mediated by IFN-λs.

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BDCA3$^+$DCs produce IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected Huh7.5.1 cells.

We stimulated freshly isolated BDCA3$^+$DCs, pDCs and mDCs with infectious viruses, such as cell-cultured HCV (HCVcc), Japanese encephalitis virus (JEV) and herpes simplex virus (HSV). In preliminary experiments, we confirmed that HCVcc stimulated BDCA3$^+$DCs to release IL-28B in a dose-dependent manner **(Fig S5).** BDCA3$^+$DCs produced a large amount of IL-28B upon exposure to HCVcc and released a lower amount of IFN-α upon HCVcc or HSV **(Fig 4A)**. In contrast, pDCs produced a large amount of IFN-α in response to HCVcc and HSV and a much lower level of IL-28B upon HCVcc **(Fig S6).** In mDCs, IL-28B and IFN-α were not detectable with any of these viruses (data not shown).

BDCA3$^+$DCs produced significantly higher levels of IL-28B than the other DCs upon HCVcc stimulation **(Fig 4B)**. By contrast, HCVcc-stimulated pDCs released significantly larger amounts of IFN-β and IFN-α than the other subsets **(Fig 4B)**. Liver BDCA3$^+$DCs were capable of producing IL-28B in response to HCVcc **(Fig 4C)**. These results show that, upon HCVcc stimulation, BDCA3$^+$DCs produce more IFN-λs and pDCs release more IFN-β and IFN-α than the other DC subsets, respectively. Taking a clinical impact of IL-28B genotypes on HCV

eradication into consideration, we focused on IL-28B/IFN-λ3 as a representative for IFN-λs in the following experiments.

In a co-culture with JFH-1-infected Huh7.5.1 cells, BDCA3$^+$DCs profoundly released IL-29, IL-28A and IL-28B **(Fig 4D, the results of IL-29 and IL-28A, not shown)**. Whereas, BDCA3$^+$DCs failed to respond to Huh7.5.1 cells lacking HCV/JFH-1, showing that IL-28B production from BDCA3$^+$DCs is dependent on HCV genome **(Fig 4D)**. In the absence of BDCA3$^+$DCs, IL-28B is undetectable in the supernatant from JFH-1-infected Huh7.5.1 cells, demonstrating that BDCA3$^+$DCs, not HCV-replicating Huh7.5.1 cells, produce detectable amount of IL-28B **(Fig 4D)**. In the co-culture, BDCA3$^+$DCs comparably released IL-28B either in the presence or the absence of transwells, suggesting that cell-to-cell contact between DCs and Huh7.5.1 cells is dispensable for IL-28B response **(Fig 4E)**. In parallel with the quantity of IL-28B in the co-culture, ISG15 was significantly induced only in JFH-1-infected Huh7.5.1 cells co-cultured with BDCA3$^+$DCs **(Fig 4F)**. A strong induction was observed with other ISGs in JFH-1-infected Huh7.5.1 in the presence of BDCA3$^+$DCs, such as IFIT1, MxA, RSD2, IP-10 and USP18 (**Fig S7**). The results clearly show that BDCA3$^+$DCs are capable of producing large

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amount of IFN-λs in response to cellular or cell-free HCV, thereby inducing various ISGs in bystander liver cells.

CD81 and endosome acidification are involved in IL-28B production from HCV-stimulated BDCA3^{+}DCs, but HCV replication is not involved.

It is not known whether HCV entry and subsequent replication in DCs is involved or not in IFN response (18, 19). To test this, BDCA3^{+}DCs were inoculated with UV-irradiated, replication-defective HCVcc. We confirmed that UV-exposure under the current conditions is sufficient to negate HCVcc replication in Huh7.5.1 cells, as demonstrated by the lack of expression of NS5A after inoculation (data not shown). BDCA3^{+}DCs produced comparable levels of IL-28B with UV-treated HCVcc, indicating that active HCV replication is not necessary for IL-28B production **(Fig 5A)**.

We next examined whether or not the association of HCVcc with BDCA3^{+}DCs by CD81 is required for IL-28B production. It has been reported that the E2 region of HCV structural protein is associated with CD81 on cells when HCV enters susceptible cells (13, 20). We confirmed that all DC subsets express CD81, the degree of which was most significant on

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BDCA3$^+$DCs **(Fig 1B, Fig S1)**. Masking of CD81 with Ab significantly impaired IL-28B production from HCVcc-stimulated BDCA3$^+$DCs in a dose-dependent manner **(Fig 5A, Fig S8)**, suggesting that HCV-E2 and CD81 interaction is involved in the induction. The treatment of poly IC-stimulated BDCA3$^+$DCs with anti-CD81 Ab failed to suppress IL-28B production **(Fig 5B)**.

HCV enters the target cells, which is followed by fusion steps within acidic endosome compartments. Chloroquine and bafilomycin A1 are well-known and broadly used inhibitors of endosome TLRs, which are reported to be capable of blocking TLR3 response in human monocyte-derived DC (21, 22). In our study, the treatment of BDCA3$^+$DCs with chloroquine, bafilomycin A1 or NH4Cl significantly suppressed their IL-28B production either in response to HCVcc or poly IC **(Fig 5A, 5B, NH4Cl,** data not shown). These results suggest that the endosome acidification is involved in HCVcc- or poly IC-stimulated BDCA3$^+$DCs to produce IL-28B. The similar results were obtained with HCVcc-stimulated pDCs for the production of IL-28B **(Fig S9)**. We validated that such concentration of chloroquine (10μM) and bafilomycin A1 (25nM) did not reduce the viability of BDCA3$^+$DCs **(Fig S10)**.

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BDCA3$^+$DCs produce IL-28B in response to HCVcc by a TRIF-dependent mechanism,

TRIF/TICAM-1, a TIR domain-containing adaptor, is known to be essential for the TLR3-mediated pathway (23). In order to elucidate whether TLR3-dependent pathway is involved or not in IL-28B response of BDCA3$^+$DCs, we added the cell-permeable TRIF-specific inhibitory peptide (Invivogen) or the control peptide to poly IC- or HCVcc-stimulated BDCA3$^+$DCs. Of particular interest, the TRIF-specific inhibitor peptide, but not the control one, significantly suppressed IL-28B production from poly IC- or HCVcc-stimulated BDCA3$^+$DCs **(Fig 6A, 6B)**. In clear contrast, the TRIF-specific inhibitor failed to suppress IL-28B from HCVcc-stimulated pDCs **(Fig 6C)**, suggesting that pDCs recognize HCVcc in an endosome-dependent but TRIF-independent pathway. These results show that BDCA3$^+$DCs may recognize HCVcc by way of TRIF-dependent pathway to produce IL-28B. .

BDCA3$^+$DCs in subjects with IL-28B major genotype produce more IL-28B in response to HCV than those with IL-28B minor type.