Sources and Signals Regulating Type I Interferon Production: Lessons Learned from Cytomegalovirus

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Type I interferons (IFN- $\alpha\beta$) are pleiotropic cytokines critical for antiviral host defense, and the timing and magnitude of their production involve a complex interplay between host and pathogen factors. Mouse cytomegalovirus (a β -herpesvirus) is a persistent virus that induces a biphasic IFN- $\alpha\beta$ response during the first days of infection. The cell types and molecular mechanisms governing these 2 phases are unique, with splenic stromal cells being a major source of initial IFN- $\alpha\beta$, requiring communication with B cells expressing lymphotoxin, a tumor necrosis factor family cytokine. Here we review the factors that regulate this lymphotoxin-IFN- $\alpha\beta$ “axis” during cytomegalovirus infection, highlight how stroma-derived IFN- $\alpha\beta$ contributes in other models, and discuss how deregulation of this axis can lead to pathology in some settings.

Introduction

THE TYPE I INTERFERONS (IFN- $\alpha\beta$) and tumor necrosis factor (TNF) family cytokines are critical for mounting effective immune defenses. In turn, deregulated expression of these cytokines can promote autoimmunity, highlighting their importance in maintaining immune homeostasis. The significance of IFN- $\alpha\beta$ and TNF-related ligands in antiviral immunity is supported by the fact that most viruses have evolved counterstrategies to modulate their signaling. The herpesviruses are prime examples of this, where extensive coevolution with their hosts has resulted in the establishment of lifelong persistence/latency in the face of a robust immune response. The cytomegalovirus (CMV) infection model (a β -herpesvirus) provides an excellent opportunity to probe for mechanisms that regulate antiviral defenses, as >3% of the mouse genome is predicted to regulate immunity to this virus (Beutler and others 2005). CMV is particularly well suited for exploring immune defenses that are impacted by homeostatic signaling networks, because this virus establishes largely asymptomatic equilibrium with its immune-competent host. The lymphotoxin $\alpha\beta$ (LT $\alpha\beta$)-LT β receptor (LT β R) cytokine system is a master regulator of a multidimensional “immune circuit,” signaling both constitutively and inducibly to regulate stromal and hematopoietic cells in various tissues. Here we will discuss how the CMV model first exposed a role for LT $\alpha\beta$ signaling in the regulation of IFN- $\alpha\beta$ production by stromal cells and how this LT $\alpha\beta$ -IFN- $\alpha\beta$ “axis” may regulate other immune and/or disease processes.

Cytomegalovirus

Human CMV (HCMV) infects the majority of population (50%–90%) (Staras and others 2006), is usually acquired early in life, and is largely asymptomatic in healthy individuals. However, in persons with underdeveloped or compromised immunity, primary or reactivated HCMV infection can lead to serious disease (eg, congenital infection) (Pereira and others 2005). The robust reactivation of CMV from latency upon immune suppression highlights the need for constant immune surveillance to maintain homeostasis between this virus and its host over a lifetime of infection. Host-specific replication/evolution of the CMVs has hindered their study in heterologous animal models, and consequently, the mouse model of CMV (MCMV) infection is commonly used. MCMV displays similar tissue tropism, temporal regulation of gene expression, and pathogenesis to HCMV and has been an invaluable model for dissecting various mechanisms regulating immune control of CMV. Additionally, the rat, guinea pig, and rhesus monkey models of CMV infection have provided valuable immunological data (Kern 2006; Powers and Fruh 2008). It is notable that sequence divergence between CMVs is concentrated largely in the “nonessential” open reading frames (ORFs) that function mainly as immune modulators, highlighting how antiviral defenses drive host-specific CMV evolution. Consequently, studying the CMV–host relationship has yielded unique insight into the mechanisms that promote and maintain immune homeostasis over a lifetime of infection.

The Innate Immune Response Early During CMV Infection

IFN- $\alpha\beta$ and natural killer (NK) cells are critical for innate control during the first few days of MCMV infection (Scalzo and others 2007). Early studies examined how IFN- $\alpha\beta$ signaling regulates MCMV replication, both *in vitro* and *in vivo* (Osborn and Medearis 1966; Kern and others 1978; Grundy and others 1982; Chong and others 1983). It is difficult to dissect NK cell versus IFN- $\alpha\beta$ functions, because in addition to acting directly on infected cells, IFN- $\alpha\beta$ is critical for activating NK cell effector functions, promoting their cytotoxicity and proliferation/survival via signal transducer and activate of transcription (STAT1) and interleukin (IL)-15-dependent pathways (Orange and Biron 1996; Nguyen and others 2002). IFN- $\alpha\beta$ is detectable in the serum at high levels as early as 6 h following MCMV infection (Grundy and others 1982) and peaks at 8–12 h before decreasing (Schneider and others 2008). There is a second wave of IFN- $\alpha\beta$ production at ~36–48 h detectable in organs and circulation, commensurate with high levels of IL-12, and this occurs in response to the first round of MCMV spread *in vivo* (Schneider and others 2008). Work by Biron and colleagues revealed that IL-12 is derived primarily from dendritic cells (DC) at this time, which then promotes STAT4-dependent IFN- γ production by NK cells, a pathway functionally separable from IFN- $\alpha\beta$ -STAT1 NK cell cytotoxicity (Orange and others 1995; Dalod and others 2002; Nguyen and others 2002). Invariant NK-T cells also secrete high levels of IFN- γ at 36 h, requiring both IL-12 and IFN- $\alpha\beta$ for maximal production (Tyznik and others 2008; Wesley and others 2008). Interestingly, invariant NK-T cells upregulate CD69 during the first phase of the innate cytokine response (~12 h), but do not produce IFN- γ at this time (Tyznik and others 2008). In this review, we will primarily focus on the LT $\alpha\beta$ -dependent pathways that regulate the initial phase of the IFN- $\alpha\beta$ response to MCMV infection derived from splenic stromal cells.

Stromal Cells as Targets of MCMV Infection and Producers of IFN- $\alpha\beta$

At ~8 h after MCMV infection, IFN- α and IFN- β production peaks in the spleen, liver, and serum (Schneider and others 2008). The levels then return to near background by 24 h, surging again at ~36–48 h. MCMV requires ~30 h to complete its replication cycle, and therefore, IFN- $\alpha\beta$ production at 8 h is derived from cells responding to the primary virus inoculum, with the second phase occurring in response to the first round of MCMV spread/production *in vivo*. Physical separation of splenic stromal and hematopoietic cells at 8 h after infection revealed the stroma to be the major source of IFN- $\alpha\beta$ and also the main cellular compartment where MCMV initially replicates (Schneider and others 2008). In contrast, the second phase of IFN- $\alpha\beta$ production is derived primarily from DC (Dalod and others 2003), with contributions from both plasmacytoid DC and conventional DC (reviewed in Marshall and Geballe 2009; Loewendorf and Benedict 2010).

These results pointed to a lymphoid tissue stromal cell being a key regulator of the primary innate cytokine response to MCMV infection, but what is the nature of this stromal cell? Stromal cells help to maintain the 3-dimensional integrity and microarchitecture of lymphoid

tissues, forming a basement structure where lymphocytes and antigen-presenting cells adhere, traffic, and congregate. Although stromal cell subsets are not nearly as well characterized as their hematopoietic counterparts, significant diversity does exist within this population (Kraal and Mebius 2006). Splenic stromal cell subsets are often identified based on their physical location [marginal zone (MZ), red pulp, or white pulp], expression of integrins and lectins (eg, MAdCAM-1, VAdCAM-1, BP-3, gp38/podoplanin), and production of specific homeostatic chemokines (eg, CCL21 and CXCL13) (Mebius and Kraal 2005; Mueller and Germain 2009). However, they have remained somewhat enigmatic because of the many technical challenges associated with isolating and culturing them under conditions where they maintain their phenotype(s). For example, fibroblast reticular cells that normally reside in the T-cell zone continue to express several identifiable markers when cultured *ex vivo* (gp38, VCAM, ER-TR7), but they do not maintain expression of their “signature chemokine,” CCL21 (Kataikai and others 2004; Link and others 2007). However, if these same cells are grown on a 3-dimensional matrix and subjected to high fluid flow rates, then CCL21 mRNA expression is induced (Tomei and others 2009). In short, this example highlights the complexities associated with trying to dissect the nature of the stromal cell(s) that respond to initial MCMV infection, or any other pathogen for that matter, as purifying stromal cell subsets and subjecting them to *ex vivo* analysis is not a straightforward task.

Recently, the location of the splenic stromal cells targeted by MCMV has been identified (Hsu and others 2009) (Fukuyama, Verma, Benedict, and Ware, unpublished observations). Cells infected by GFP-expressing MCMV are localized almost exclusively to the splenic MZ at 8 h. The MZ contains several specialized populations of stromal cells, one being marginal zone reticular cells (MRCs), which are phenotypically similar to fibroblast reticular cells dispersed throughout the spleen (Kraal and Mebius 2006). MRCs can be distinguished from MZ sinus endothelial cells by their CD31[−]/ER-TR7⁺ expression (Kraal and Mebius 2006). MRCs express various adhesion molecules (eg, MAdCAM-1, VCAM-1, and ICAM-1) that bind integrins on hematopoietic cell subsets (eg, MZ B cells, macrophage populations), preferentially maintaining these cells in the MZ. MRCs also form bridging channels and conduits, the structural “highways” that facilitate trafficking of hematopoietic cells and small proteins from the MZ to locales in the red and white pulp (Mebius and Kraal 2005; Zindl and others 2009). As the majority of circulating blood enters the spleen through the MZ sinus, MRCs are well positioned to function as first responders to pathogens or foreign antigens.

The first cells infected by MCMV in the MZ are likely MRCs, as they express CD29, MAdCAM-1, and ER-TR7. Initial infection of MRCs by MCMV appears very specific, not infecting closely juxtaposed endothelial cells or macrophages (Hsu and others 2009). Interestingly, recent work indicates that lymphoid tissue reticular cells may regulate peripheral T-cell tolerance (Fletcher and others 2010). Given that the same MRCs likely harbor latent CMV (Mercer and others 1988), it is tempting to speculate that CMV infection of these cells might impact tolerance. In this vein, CMV is frequently discussed as a potential cofactor in autoimmune disorders (Varani and others 2009), but direct causative proof in most cases has been difficult to obtain because of the high prevalence of CMV infection in the general population.

LT α β -LT β R Regulation of Lymphoid Tissue Stroma

LT α β -LT β R signaling impacts the development and/or microarchitecture of all lymphoid tissues (Ware 2005) (Fig. 1). Its requirement for lymph node (LN) development was first revealed in *lt α* -deficient mice (LT α $^{-/-}$), a phenotype recapitulated in LT β R $^{-/-}$ and LT β $^{-/-}$ mice (reviewed in Tumanov and others 2003b). LN development halts during embryogenesis because of a requirement for cross-talk between LT β R-expressing stromal cells and LT α β -expressing lymphoid tissue inducer cells in the developing LN anlagen (Randall and others 2008). In contrast to LN, the spleen still forms in the genetic absence of LT α β -LT β R signaling, but the development and/or differentiation of stromal cells in both the MZ and white pulp is altered (Tumanov and others 2003a; Zindl and others 2009). Blocking LT α β -LT β R signaling in both neonates and adults has also revealed its requirement for the homeostatic maintenance of splenic stromal cells postembryogenesis (Ettinger and others 1996; Mackay and others 1997). In total, the role that LT α β -LT β R signaling plays in the regulation of lymphoid tissue stroma at the developmental and homeostatic level is complex and multifaceted. Importantly, however, the many available genetic and pharmacologic tools provided a unique opportunity to assess its role in mounting host defenses to CMV, a persistent virus that targets lymphoid tissue stromal cells.

Making the Link Between Lymphotoxin and IFN- α β During HCMV Infection *In Vitro*

Initial studies *in vitro* suggested a role for LT α β -LT β R signaling in regulating type I IFN during CMV infection. Triggering LT β R signaling during the first hours of HCMV

infection in cultured fibroblasts dramatically amplifies IFN- β production (~ 10 – 100 -fold, varying upon the multiplicity of infection (MOI) of infection) (Benedict and others 2001). This is not unique to LT α β -LT β R signaling, as LT α -TNF receptor (TNFR) signaling also enhanced IFN- β production. In addition, IL-2-activated human NK cells can function as “sources” of LT β R and TNFR ligands, similarly enhancing IFN- β production from HCMV-infected stromal cells, even at very low effector:target ratios (Iversen and others 2005). In all these scenarios, HCMV infection and LT β R/TNFR function cooperatively to induce IFN- β , with receptor signaling alone inducing no IFN- β and virus alone being a weak inducer. However, the 2 added commensurately, or within a few hours, induced high levels of IFN- β transcription and secretion, which inhibited viral spread in the cultures by a non-cytolytic, reversible mechanism (Benedict and others 2001). Subsequent work from several groups has shown that HCMV imposes a direct, viral transcription-dependent block to early IFN- β transcription (Browne and others 2001), mediated, at least in part, by the immediate early-2 protein (Taylor and Bresnahan 2005, 2006; Jarvis and others 2006). The immediate early-2 block to IFN- β is thought to involve inhibition of NF κ B, a key component of the IFN- β transcriptional enhancer (Maniatis and others 1998). Notably, NF κ B activation is absolutely required for LT β R/TNFR signaling to cooperatively induce IFN- β transcription in HCMV-infected cells (Benedict and others 2001). Therefore, we would propose a model where if an extrinsic activator of NF κ B (ie, LT β R, TNFR, etc.) signals concurrently upon initial HCMV infection of a stromal cell, this can circumvent an HCMV-imposed block to IFN- β transcription. Consequently, multiple factors must be considered when assessing the innate cytokine response during CMV infection, as both the host signaling pathways and viral countermeasures impact its magnitude.

FIG. 1. Lymphotoxin and the immediate tumor necrosis factor (TNF) family. The TNF family ligands bind their cognate receptors as soluble and/or cell-associated trimers, shown by the *arrows*. LT α 1 β 2 and LIGHT bind the LT β R, recruiting TNF receptor-associated factor (TRAF) adaptors to the receptor and resulting in the liberation/stabilization of NF κ B-inducing kinase (NIK). NIK then activates IKK α and the downstream noncanonical NF κ B signaling pathway. Studies indicate that LT α β -LT β R and NIK-dependent signaling contributes to initial interferon (IFN)- α β production by splenic stromal cells during mouse cytomegalovirus (MCMV) infection.

The LT-IFN- $\alpha\beta$ Axis *In Vivo* During MCMV Infection

Although the results with HCMV-infected fibroblasts suggested the existence of an LT-IFN- $\alpha\beta$ axis operable in stromal cells, this needed to be examined *in vivo*. Initial studies found that mice deficient for LT $\alpha\beta$ -LT β R signaling were more sensitive to MCMV-induced death (Benedict and others 2001). Subsequent studies revealed that LT $\alpha^{-/-}$, LT $\beta^{-/-}$, and LT β R $^{-/-}$ mice could not control acute MCMV replication (Banks and others 2005). Intriguingly, at high doses of MCMV infection, a dramatic apoptotic collapse of splenic lymphocytes and DC occurs at 2–3 days after infection in mice lacking LT $\alpha\beta$ -LT β R signaling. A similar massive splenocyte death is observed in mice lacking the type I IFN receptor (IFN- $\alpha\beta$ R), again highlighting a parallel between these 2 innate cytokine systems. Induction of IFN- β mRNA was dramatically reduced in the spleen of LT $\alpha^{-/-}$ and LT $\beta^{-/-}$ /LIGHT $^{-/-}$ mice at 8 h after MCMV infection (Schneider and others 2008). Injection of an agonistic anti-LT β R antibody restores IFN- β mRNA levels to a large degree in these genetically deficient mice and rescues the vast majority of splenocyte apoptosis, but cannot amplify the type I IFN response in wild-type mice. Taken together, these data suggest the existence of a coordinately regulated innate defense pathway requiring both lymphotoxins and IFN- $\alpha\beta$ to protect the homeostasis of lymphoid tissue upon infection with a normally nonpathogenic herpesvirus.

Lymphotoxin-Dependent Cross-Talk Between B cells and Stromal Cells Promotes the IFN- $\alpha\beta$ Response to MCMV

Results with bone marrow chimeric mice suggested that the MCMV-infected, IFN- $\alpha\beta$ -producing splenic stromal cells must express the LT β R themselves (Fig. 2) (Schneider and others 2008). Therefore, if the stroma must express the LT β R, what cell type provides the LT $\alpha\beta$? LT $\alpha\beta$ can be expressed by both naive and activated B and T lymphocytes, suggesting either cell type could be responsible. Accordingly, mice lacking all B cells display a dramatic deficiency in the initial

IFN- $\alpha\beta$ response to MCMV, whereas mice lacking T cells are normal (Schneider and others 2008). Further, conditional knockout mice lacking *lt β* expression specifically in either B or T cells confirmed that it is LT β -expressing B cells that communicate with the LT β R-expressing MRCs to promote the initial type I IFN response in the spleen (Schneider and others 2008).

Organ-Specific Regulation of IFN- $\alpha\beta$ Production by Lymphotoxin Signaling

Although cross-talk between B cells and stroma via the LT $\alpha\beta$ -LT β R pathway is crucial for initial IFN- $\alpha\beta$ production in the spleen during MCMV infection, initial type I IFN production in the liver is not dependent upon LT β R signaling (Schneider and others 2008). The mechanism promoting initial IFN- $\alpha\beta$ production in liver is currently unknown, but liver sinusoidal endothelial cells and hepatocytes are primary targets of initial MCMV infection in this organ (Sacher and others 2008). Although both these cell types can express LT β R, perhaps the absence of a significant, resident B-cell population in the liver excludes the role of this axis during initial MCMV infection. Interestingly, the relative amount of IFN- $\alpha\beta$ produced by the liver compared with the spleen is significantly lower when normalized to MCMV gene expression (Schneider and others 2008), suggesting that splenic MZ stromal cells are robust producers of IFN- $\alpha\beta$ on a per cell basis when encountering this virus. Additionally, as mice deficient in LT β R signaling show normal initial levels of IFN- $\alpha\beta$ production in the liver but show major defects in the serum, this strongly suggests that the spleen is a major contributing organ to systemic IFN- $\alpha\beta$ levels during MCMV infection.

LT β R Signaling Pathways That Regulate the Stromal IFN- $\alpha\beta$ Response

Upon binding of the LT β R by LT $\alpha\beta$ or LIGHT, the TNFR-associated factors (TRAFs) function as cytoplasmic signaling adaptors (Fig. 1) (Nakano and others 1996; VanArsdale and others 1997). Activation of the noncanonical NF κ B pathway

FIG. 2. LT $\alpha\beta$ -expressing B cells cross-talk with splenic stromal cells to promote initial type I IFN production during MCMV infection. Upon entry into the spleen, MCMV first infects ERTR-7 $^{+}$ MAdCAM-1 $^{+}$ CD29 $^{+}$ marginal zone reticular cells (MRCs), and these stromal cells are the likely source of the majority of type I IFN produced locally and detected systemically during initial infection. B cells that constitutively express surface LT $\alpha\beta$ provide a required signal to LT β R-expressing stromal cells to induce type I IFN. The pattern recognition receptor (PRR) detecting MCMV in the stroma is currently unknown.

occurs after recruitment of TRAF2 and TRAF3 to the LT β R upon ligand binding, competitively displacing the NF κ B-inducing kinase (NIK) from these TRAFs, which constitutively degrade NIK via their E3 ubiquitin ligase activity in unactivated cells (Sanjo and others 2010), ultimately resulting in activation of IKK α and RelB-dependent transcription (Dejardin and others 2002). Mice with a naturally occurring mutation in NIK (*aly/aly*) also show a severe defect in the MCMV-induced initial IFN- $\alpha\beta$ response (Schneider and others 2008), indicating that the LT β R-NIK signaling pathway operates in MZ stromal cells to modulate innate defenses.

Mice lacking Toll-like receptor (TLR) signaling (MyD88/TRIF deficient) mount a normal initial IFN- $\alpha\beta$ response to MCMV infection, distinguishing the LT β R from TLR-dependent pathways in regulating MCMV defenses in MZ stromal cells (Schneider and others 2008). The TLR-independent pathways that regulate innate sensing of DNA viruses are not as well defined as they are for RNA viruses; however, significant recent advances have been made (Pichlmair and Reis e Sousa 2007; Tamura and others 2008; Takeuchi and Akira 2009). The DNA-dependent activator of interferon response factors (IRFs) (DAI/DLM-1/ZBP1) senses dsDNA in the cytoplasm (Takaoka and others 2007). Dependent activator of IRF (DAI) can bind the receptor-interacting protein (RIP) 1 and RIP3 through its RIP homotypic interacting motif (Kaiser and others 2008; Rebsamen and others 2009) and transmit downstream signals through Cardif (IPS-1/MAVS/VISA) and/or the stimulator of IFN genes/mediator of IRF3 activation to activate IFN- β transcription (Ishikawa and Barber 2008; Takeuchi and Akira 2008; Yanai and others 2009). Although the sensor/pathway regulating innate recognition of MCMV remains unidentified, a recent report identifies DAI as necessary and sufficient for IRF3 activation and IFN- β production during HCMV infection of cultured fibroblasts (DeFilippis and others 2010). As IRF3 and NF κ B are both essential components of the IFN- β -enhancesome (Thanos and Maniatis 1995), it is enticing to speculate that LT β R regulation of type I IFN production may synergize with a DAI-dependent (or DAI-like) pathway in some cell types.

How the Lessons Learned from Studying LT $\alpha\beta$ -LT β R Regulation of Innate Defense to CMV May Extend to Other Systems

Stromal cells are major producers of IFN- $\alpha\beta$ during MCMV infection, but do they contribute in other contexts? Recent studies with the ssRNA Chikungunya virus (CHIKV) identified the stroma as producing IFN- $\alpha\beta$ during infection (Schilte and others 2010). Similar to several other RNA viruses, a Cardif-dependent recognition pathway is operable in fibroblasts, but whether LT $\alpha\beta$ -dependent signals contribute to CHIKV innate defenses remains to be determined. Recent work has also shown that splenic stromal cells regulate the effectiveness of DC-targeted vaccines (Longhi and others 2009). The adjuvant activity of poly-IC in this system was ascribed primarily to its induction of type I IFN *in vivo*. Similar to studies with MCMV and CHIKV, a bone marrow reconstitution approach revealed radioresistant stromal cells to be the major MDA5-dependent producers of IFN- $\alpha\beta$ after poly-IC injection, which then functioned to mature vaccine antigen-targeted, DEC-205+ DC and promote the adaptive response.

As a flip side to the protective role of LT $\alpha\beta$ -LT β R signaling in promoting antiviral innate defenses, deregulated and/or extended activation of this pathway has recently been shown to promote tumorigenesis. In the case of hepatitis B and C virus-associated hepatocellular carcinoma, infiltration of LT $\alpha\beta$ -expressing lymphocytes can signal to LT β R-expressing hepatocytes to drive chronic hepatitis and liver cancer (Haybaeck and others 2009). In turn, expression of LT $\alpha\beta$ by tumor-resident B cells drives the pathogenesis associated with castration-resistant prostate cancer (Ammirante and others 2010). In both these mouse tumor models, treatment with LT β R-Fc (a pharmacological blocker of LT $\alpha\beta$ and LIGHT signaling) restricted tumor progression. Additionally, activation of both the canonical and noncanonical NF κ B pathways downstream of LT β R activation was implicated in tumorigenesis. Consequently, these cases serve as another example that tight regulation of LT $\alpha\beta$ -LT β R signaling is critical for maintaining immune homeostasis and that chronic signaling during prolonged inflammation can disrupt this equilibrium and promote disease.

Conclusions

Although we have mainly discussed the host mechanisms regulating CMV induction of IFN- $\alpha\beta$, CMV dampens both IFN- $\alpha\beta$ induction and downstream signaling at various levels, and this has been recently reviewed (DeFilippis 2007; Lenac and others 2008; Marshall and Geballe 2009). It is critical to consider this fact when formulating any models regarding the functional consequences of IFN- $\alpha\beta$ signaling on the course of CMV infection. Additionally, the direct regulation of innate defenses by the LT $\alpha\beta$ -LT β R signaling pathway is almost certainly intertwined with its role in maintaining lymphoid tissue architecture. CMV selectively disrupts this architecture upon acute infection, but LT β R-dependent signaling contributes to its rapid restoration (Benedict and others 2006), highlighting a key difference between CMV and nonpersistent viruses, which disrupt tissue architecture for much longer periods of time (Mueller and others 2007; Scandella and others 2008). These concepts, in addition to the role that B cells can play in regulating innate antiviral defenses, will continue to be active areas of research that bridge diverse fields.

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