Transcriptome and epigenome profile of B cells in patients with Transient Hypogammaglobulinemia of Infancy

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Hypothesis

Patients with Transient Hypogammaglobulinemia of Infancy (THI) will exhibit delayed loss of methylation in B cell lymphopoiesis genes, resulting in a deficiency of mature B cell subpopulations.

1 Background

Antibodies are a vital component of the adaptive immune system. The production of antibodies occurs when naïve B cells are activated in response to foreign antigens[1]. After birth the maternal antibodies are degraded before infant B cells can mature, leading to a decline in serum antibody levels[2–4]. Physiological hypogammaglobulinemia refers to the point when serum antibody concentration reaches its lowest level, commonly at four to six months of age[5]. THI is a disorder where regular hypogammaglobulinemia is prolonged or exacerbated then spontaneously alleviated.[5–14]. The mechanism causing THI is at present unknown[6].

Cause of THI

Studies investigating THI have not identified any deficiencies in the antibody production pathway. Levels of circulating B cells have been reported as normal with subpopulations of B cells also intact[5,11,13–16]. Furthermore, upon antigenic challenge, most patients produce

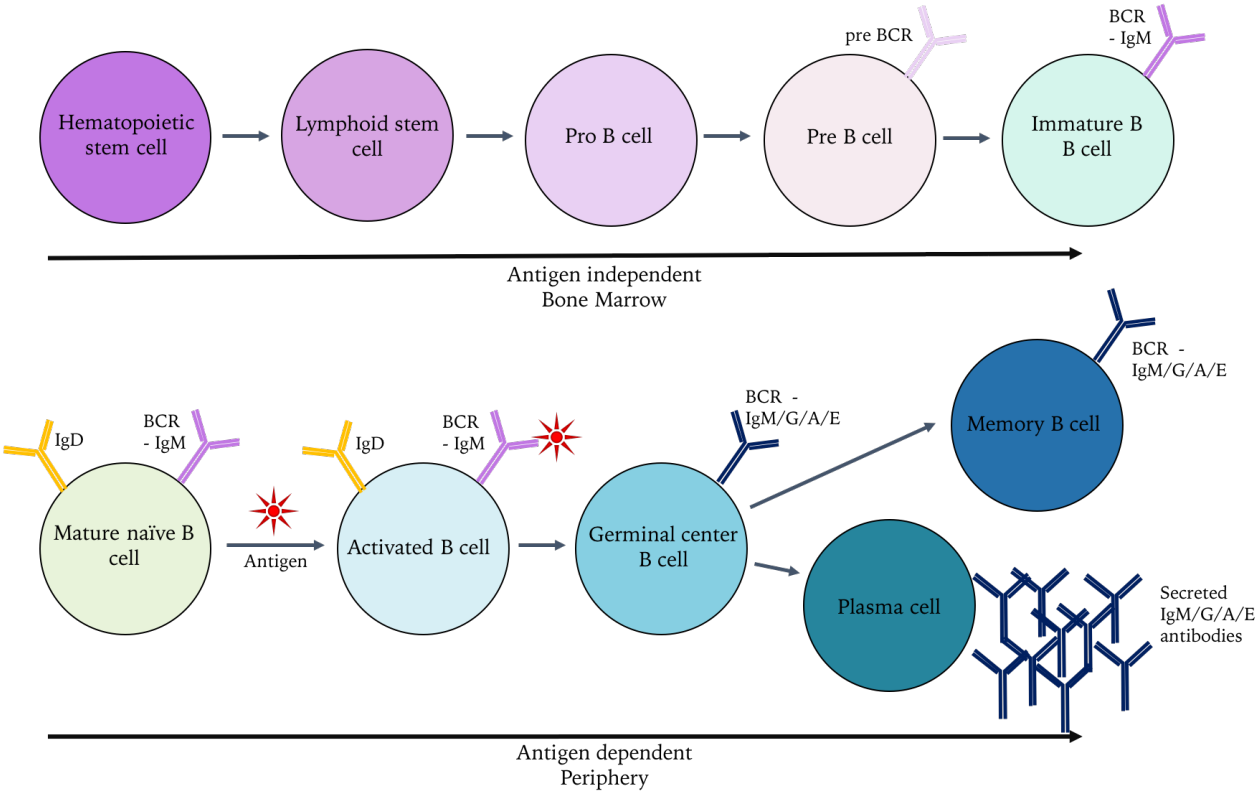


Figure 1: B cell development from hematopoietic stem cell to memory and plasma cells. Phases are shown as antigen independent or dependent and location (bone marrow or periphery) is indicated. IGM/G/A/E indicates option of antibody isotype. BCR: B cell receptor.

a normal antibody response[5,10–14]. THI was initially thought to be hereditary[17,18], however following studies have not shown supporting evidence[13,16,19]. With apparently normal B cell populations and no genetic basis, the cause of THI has been extensively speculated, but no proposed mechanism has been supported by replicated evidence[9,10,13,15–21]. The most agreed upon cause is a delay in B cell maturation or activation[10,11,22,23].

B lymphocytes develop in the bone marrow from hematopoietic precursors[24]. Development and maturation progress through stages labelled in figure 1. Plasma cells produce the antibodies required for humoral immunity. Failure to proceed at any stage of B cell development can result in a deficiency of plasma cells and thence antibody deficiency.

B lymphocyte differentiation is tightly regulated by transcription factors (TFs). Figure 2 shows the complex network of auto-regulation, cross regulation, and positive and negative

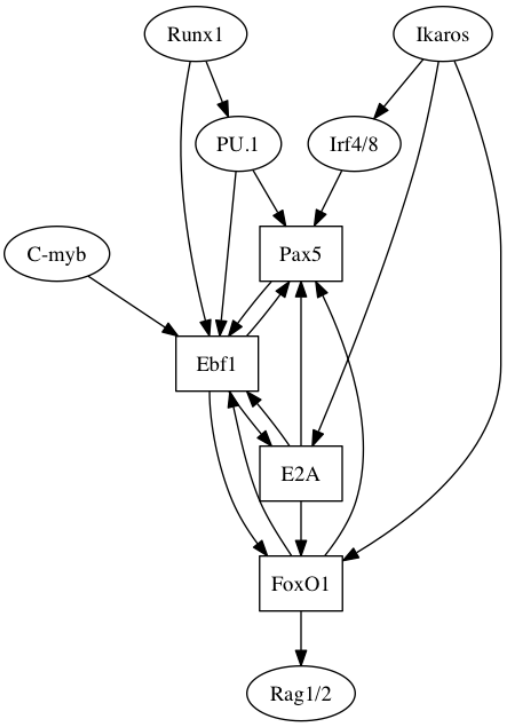


Figure 2: Schematic representation of interactions between early B cell transcription factors. Adapted from Choukrallah and Matthias[26]. Arrows indicate positive regulation, direct positive regulation is indicated by reciprocal arrows.

feedback loops[26–33]. TFs prominent in early stages of B cell specification and commitment include Pax5, E2A, Ebf1, Ikaros, PU.1, and FoxO1[33–40]. Loss of function studies involving these TFs show some defect in B cell lineage commitment, resulting in a loss of a B cell subset or in some cases the entire B cell lineage[26].

Flow Cytometry

Previous investigations into THI claimed that subpopulations of B cells are the same in THI patients as controls[5,11,13–16]. The claim is supported by studies which distinguished only between mature and immature B cells. Analysis techniques have improved substantially since the most recent experimental THI study took place. In particular flow cytometry (FACS) has developed extensively, increasing the number of measured parameters per cell[41]. 18-parameter FACS is now routinely used[42], 30-parameter flow cytometers are becoming commercially available, and 50-parameter FACS is predicted to be available soon[41]. An increased number

of measurable parameters allows more comprehensive study of B cell subpopulations.

Lineage commitment

Epigenetic modifications act in concert with TFs to confer the phenotype of many cell subsets of the immune system[29,31,43]. A prominent example is the activation and differentiation of the many T cell subsets[44,45]. Confirmation of the T regulatory cell (Treg) lineage relies on stable expression of the TF FOXP3. However, FOXP3 expression occurs in all T cell subsets upon activation[27,46]. The distinguishing factor between Tregs and other subsets is the methylation status of the FOXP3 gene, otherwise known as the Treg-specific demethylated region (TSDR)[47]. When the T cell receptor is engaged in Treg progenitors, demethylation occurs at the TSDR. FOXP3 can then bind to its own gene stabilising expression, reinforcing commitment to the Treg lineage. Contrastingly, in other subsets the TSDR remains methylated and so FOXP3 expression is transient[46]. Hence methylation status of key genes can permit lineage commitment.

Aberrant epigenetics have recently been implicated as the cause of Common Variable Immunodeficiency (CVID), a disease similar to THI[48]. CVID is a late-onset primary immunodeficiency characterised by dysfunction or loss of B lymphocytes and decreased immunoglobulin production[49]. Epigenome and transcriptome analysis revealed that the Pax5 enhancer was hypermethylated and severely down-regulated. Pax5 is essential to commit a cell to the B cell identity through activation of 170 B cell specific genes and repression of at least 110 lineage inappropriate genes[50–52]. Tallmadge et al.[48] proposed that the cause of CVID was the methylation of Pax5. If the methylation of the Pax5 enhancer was silencing the gene as proposed, a decline of B lymphopoiesis would occur in the bone marrow, followed by a depletion of B cells characteristic of CVID.

It is possible that the prolonged antibody deficiency exhibited in THI patients is caused by a similar mechanism of epigenetic silencing. Epigenetics encompasses an extensive list of mechanisms including small RNA regulation, DNA methylation, chromatin modifications[29,30,53,54].

DNA methylation was the first epigenetic mechanism recognised, and is the most commonly studied[44]. Hence studying methylation of THI samples is a logical first step to understanding THI.

2 Summary and experimental aims

- THI is a self-limited disorder characterised by prolonged deficient levels of serum antibody which spontaneously subside[5–15,18,55].
- The claim of intact B cell subpopulations in THI is supported by outdated methods which only distinguish mature from immature B cells, providing good reasoning for a more comprehensive study.
- Tallmadge et al.[48] provided evidence that in CVID, aberrant methylation of key B cell maturation region, Pax5, was the cause of late onset dysfunctional B lymphopoiesis.

Aim 1: Using FACS, describe the B cell subpopulations in THI patients and normal individuals throughout early development.

Aim 2: Using whole genome bisulphite sequencing, identify regions of the genome which are differentially methylated in THI samples and controls.

Aim 3: Using RNA-Seq, identify regions which are differentially expressed in THI samples and controls.

3 Data collection and management

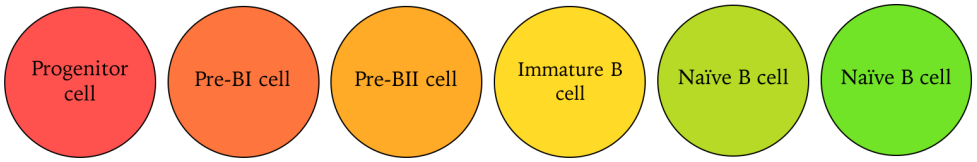
3.1 Collecting samples

Whole blood and tonsil samples from 26 THI patients has been donated from the Wolfson Medical centre Pediatric Department (table 1). Further peripheral blood, bone marrow and tonsil samples will be obtained from the Women's and Children's Hospital Immunodeficiency clinic. Blood will be taken from THI patients between initial presentation and normalisation of antibody levels. Bone marrow will be taken in six monthly intervals following presentation until normalisation of antibody levels. Control samples will come from donations by healthy infants.

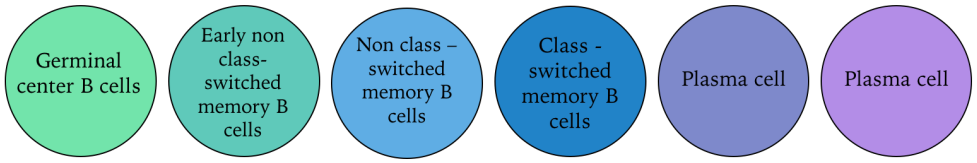
3.2 Matching controls

To determine B cell maturation changes caused by THI, it is imperative to compare samples at the closest possible developmental stages. Where possible, THI samples will be fully matched to control samples. The most important criteria to match will be age, as it is the most prominent indicator of immune system development. The method of both birth (vaginal or caesarian) and feeding (bottle or breastfed) will be considered as both have a large influence over the immune system[56–60]. Factors such as gender and ethnicity will also be matched if possible. Diets will be standardised in all new patients.

In the samples provided, listed in table 1, there are gaps in the clinical history of samples. Without information such as ethnicity or mode of birth, it is impossible to match each sample to an appropriate control. Additionally, the control samples will be taken from participants' donations which may not match the clinical history of any THI patient. While completely matching samples and controls is preferable, there are limitations in the scope of the matching.



Source	Bone marrow	Bone marrow	Bone marrow	Bone marrow	Blood	Tonsil
Surface markers	$CD34^{++}$ $CD19^{-}$	$CD34^{+}$ $CD19^{+}$	$CD34^{-}$ $CD19^{+}$ $sIgM^{-}$	$CD34^{-}$ $CD19^{+}$ $sIgM^{+}$	$CD19^{+}$ IgD^{+} $CD27^{-}$	$CD19^{+}$ IgD^{+} $CD27^{-}$



Source	Tonsil	Blood	Blood	Blood	Bone marrow	Tonsil
Surface markers	$CD19^{+}$ $CD20^{++}$ $CD38^{+}$	$CD19^{+}$ $CD27^{-}$ $CD23^{-}$ Rhodamine 123^{+}	$CD19^{+}$ $CD27^{+}$ IgM^{+} IgD^{+}	$CD19^{+}$ IgG^{+} $CD27^{+}$	$CD138^{+}$	$CD19^{+}$ $CD20^{+}$ $CD38^{++}$

Figure 3: Description of the FACS sorting markers used to segregate B cell populations.

4 Aim 1: Using FACS, describe the B cell subpopulations in THI patients and normal individuals throughout early development

Hypothesis: THI samples will be deficient in mature B cell subsets.

4.1 Proposed experiment

To analyse the distribution of B cell subpopulations, we will segregate the B cells using flow cytometry as in Kulis et al.[32] and Oakes et al.[28]. B cell populations obtained will include progenitor, pre-BI, pre-BII, immature and plasma cells from bone marrow, naïve and memory B cells from peripheral blood and plasma cells and germinal centre and naïve B cells from tonsils. FACS will be used for all three tissues to sort populations using the surface markers in figure 3.

4.2 Possible outcomes and interpretations

Segregating samples into B cell subpopulations serves two purposes. Firstly, sorting means we can compare cells at the same maturity stage. It is imperative that any changes observed between THI and controls are caused by THI rather than normal B cell maturation changes. While there are always limitations when applying discrete stages to continuous processes, segregating B cells into such specific subtypes significantly reduces the chance of detecting differences which are due to normal development.

Secondly, as the cells will be separated based on maturity, we are able to analyse the distribution of subtypes. If there is a deficiency of mature B cells in THI samples relative to controls, it could indicate a block in the B cell development pathway, as in Tallmadge et al.[48]. Alternatively, the distribution of B cell subpopulations could be intact, as claimed previously[5,11,13–16]. If each subset is intact it would indicate that the cause of THI may not be an error in B cell development, and perhaps investigation into antibody production or degradation should follow. Therefore FACS allows us to compare cells more accurately and can show if the mechanism affects B cell development.

5 Aim 2: Using whole genome bisulphite sequencing, identify regions of the genome which are differentially methylated in THI samples and controls

Hypothesis: B cells from THI patients will exhibit hypermethylation at key B cell development regions.

5.1 Proposed experiment

To investigate global DNA methylation of THI and control samples, we will produce full methylomes of the B cell lineages in figure 3. Whole genome bisulfite sequencing (WGBS) will be used to obtain base-pair resolution of all methylated cytosines within the genome[28,32]. To perform the analysis, we will use two sets of biological replicates for each of the samples. Samples will undergo two rounds of bisulfite conversion to ensure a cytosine to thymine conversion rate of over 99%. Treated samples will be sequenced on an Illumina HiSeq 2000 platform and mapped to the genome using the STAR algorithm (v2.4.2a)[61].

We will then use ChIP-seq data from the ENCODE project[62] to analyse methylation status in the context of transcription factor binding sites. The relative enrichment of each TFBS in any differentially methylated regions will be calculated in comparison to background reads. A Fisher's exact test will be used to assign an odds ratio and P value to each comparison. Of particular focus will be the genes in figure 2 which are specific to B lymphopoiesis.

5.2 Possible outcomes and interpretations

Unbiased DNA methylation maps as in figure 4 will be produced for each of the sorted cell populations. It is expected that in both THI and control samples in all age groups, global methylation decreases as the B cells mature[32,63–65]. If THI causes delayed loss of methylation,

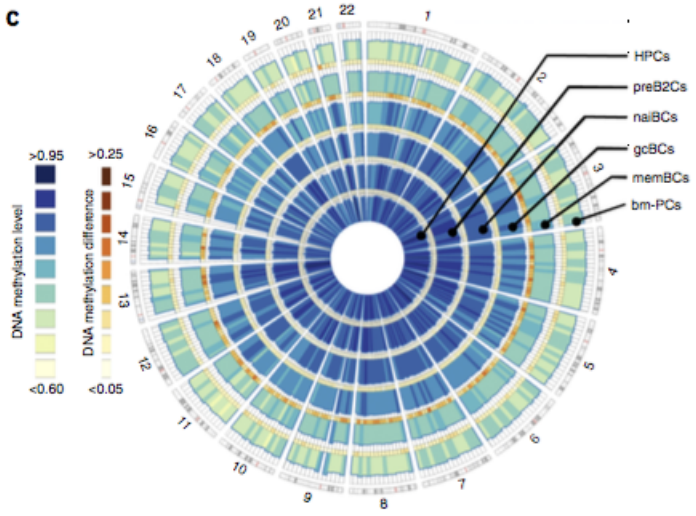


Figure 4: Methylome map produced by Kulis et al.[32].

Figure 5: Cytosine methylation status is shown for six stages of B cell development. Concentric circles are labelled with developmental stage. Colour corresponds to methylation status indicated in the scale.

it would be evident in methylation maps. By comparing the same individuals at different ages, we expect that regions of hypermethylation will gradually deplete and resemble the controls.

The ChIP-Seq data provides a more detailed view of key regions such as transcription factor binding sites (TFBSs). We will produce a heatmap which displays the correlations between TFBSs and differentially methylated regions. In the THI samples, we expect to find that regions related to B cell maturation or antibody production will be hypermethylated compared to background levels. The location of differentially methylated regions (DMR) will indicate any potential causes of THI. If a region associated with B cell maturation, such as Pax5, is found to be differentially methylated it indicates that THI could be a result of a developmental block like that seen in CVID[48]. Alternatively if there is a DMR associated with gene rearrangement, THI could be caused by a lack of B cell diversity. Any DMRs identified can be further investigated with expression analysis.

6 Aim 3: Using RNA-Seq, identify regions which are differentially expressed in THI samples and controls

Hypothesis: Samples from THI patients will exhibit decreased expression for B cell commitment associated genes compared to controls.

6.1 Proposed experiment

To investigate expression changes across the genome of THI samples, we will conduct RNA-Seq analysis on each of the B cell subsets in figure 3. RNA-Seq libraries will be generated using the TruSeq Stranded Total RNA kit (Illumina). Sequenced reads will be aligned to the genome using the STAR algorithm (v2.4.2a)[61]. qPCR analysis of gene expression will then be undertaken to quantify reads. Target gene expression will be presented relative to average expression for the housekeeping genes *GAPDH*, *ACTB* and *HPRT1*.

6.2 Possible outcomes and interpretations

Expression analysis will allow us to further investigate any DMRs found in methylation analysis as well as find other genes which are differentially expressed. Heatmaps will be generated to display the genes which change expression between THI and control samples.

We expect to see differentially expressed regions associated with B cell development or antibody production. For example, if Rag1/2 was found to have decreased expression, it could indicate that impaired gene rearrangement is causing THI, as gene rearrangement is vital for antibody diversity[26].

Genes which show hypermethylation in WGBS analysis are expected to have decreased expression in THI samples than controls. If expression changes occur in regions that are not differentially methylated, the implicated region could possibly be affected by a different

epigenetic mechanism such as a chromatin modification. Any expression changes not correlated with methylation status could be further investigated as potential causes of THI.

7 Conclusion

It is feasible that THI is caused by a delay in maturation as a result of aberrant methylation. FACS, WGBS and RNA-Seq provide a broad survey of B lymphopoiesis in THI. If THI is caused by stunted maturation, our data will show where it occurs and will indicate any affected pathways. Should the results show that methylation is not involved in THI, the FACS and transcriptome data provide avenues for further research.

8 Appendices

A Sample information

Table 1: Clinical details of patients with THI. Abbreviations: m, months; y, years; CVI, common variable immunodeficiency; -, unknown; def, d.

Individual	Tissue	Age at test	Gender	Ethnicity	Breastfeeding status	Mode of birth	Family history of PID
1	Whole Blood Tonsils	7m, 11m, 1y 7m, 2y 1m 2y 1m	Female	Caucasian	Breastfed	Vaginal	None
2	Whole Blood Tonsils	8m, 11m, 1y 3m, 1y 6m, 1y 8m, 2y 1m, 2y 4m, 2y 6m, 2y 8m 2y 6m	Male	Caucasian	Breastfed	Vaginal	None
3	Whole Blood Tonsils	9m, 1y 3m, 1y 9m, 2y 6m, 2y 9m, 3y 2m, 3y 6m 3y 2m	Male	Jewish	Bottle fed	Caesarian section	None
4	Whole Blood	10m, 1y 1m, 1y 8m	Male	Caucasian	Bottle fed	Vaginal	None
5	Whole Blood	9m, 11m, 1y 3m, 1y 5m, 1y 8m	Male	Asian	Breastfed	Vaginal	Brother with CVI
6	Whole Blood	7m, 9m, 11m, 1y 8m	Female	Jewish	Breastfed	Vaginal	None
7	Whole Blood	9m, 11m, 1y 2m, 1y 6m	Male	-	Breastfed	-	-
8	Whole Blood	7m, 9m,	Female	-	Breastfed	-	-
9	Whole Blood	7m, 9m, 11m, 1y 3m 1y 8m	Male	-	Breastfed	-	-
10	Whole Blood	7m, 9m, 11m, 1y 2m, 1y 6m	Male	-	Breastfed	-	-

Individual	Tissue	Age at test	Gender	Ethnicity	Breastfeeding status	Mode of birth	Family history of PID
11	Whole Blood	9m, 11m, 1y 1m, 1y 3m 1y 8m	Male	-	Bottle fed	-	-
12	Whole Blood Tonsils	7m, 11m, 1y 2m, 1y 11m, 2y 6m, 2y 9m, 3y 4m, 3y 7m, 4y 2y 11m	Female	Jewish	Breastfed	Vaginal	None
13	Whole Blood	9m, 11m, 1y 2m, 1y 6m, 2y 4m, 2y 8m	Male	Jewish	Bottle fed	Vaginal	None
14	Whole Blood Tonsils	9m, 1y 1m, 1y 7m, 2y 2m, 2y 9m, 3y 2m 3y 2m	Female	Caucasian	Bottle fed	Vaginal	None
15	Whole Blood	7m, 11m, 1y 1m	Male	Caucasian	Breastfed		Sister with IgA def.
16	Whole Blood	8m, 1y 1m, 1y 4m, 1y 6m	Male	-	-	Vaginal	-
17	Whole Blood	9m, 1y 2m, 1y 6m	Male	-	-	Vaginal	-
18	Whole Blood	7m, 9m, 1y 1m	Female	-	-	Caesarian section	-
19	Whole Blood	7m, 9m, 11m, 1y 2m, 1y 6m	Male	-	-	Vaginal	-
20	Whole Blood	9m, 11m, 1y 2m, 1y 6m	Male	-	-	Vaginal	-
21	Whole Blood	11m, 1y 3m, 1y 5m, 1y 8m	Male	-	-	Caesarian section	-
22	Whole Blood	7m, 9m, 11m, 1y 8m	Female	-	-	-	None
23	Whole Blood	9m, 1y 2m, 1y 6m	Male	-	-	-	None

Individual	Tissue	Age at test	Gender	Ethnicity	Breastfeeding status	Mode of birth	Family history of PID
24	Whole Blood Tonsils	7m, 9m, 1y 1m, 1y 6m, 1y 11m, 2y 1m 2y 4m	Female	-	-	-	None
25	Whole Blood	7m, 11m, 1y 3m, 1y 5m, 1y 8m	Male	-	-	-	Sister with IgA def.
26	Whole Blood	8m, 1y 1m,	Male	-	-	-	None

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