Role of CD4 Memory t and t Follicular Helper Cells in Urinary Bladder Cancer

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Abstract
Tumor infiltrating lymphocytes (TILs) are candidate as crucial biomarkers, since certain cancers have high lymphocyte infiltration that obtain significant role in specific cancer types as in colorectal cancer. In gastric cancer the risk factors of recurrence were negatively associated with activated CD4 memory T-cell (MTh) infiltration. Tfh found to have a systematized antitumor immunity, greatly predicted survival or patients' response to cancer therapy. The CD4 T-cell subsets role in tumor microenvironment (TMI) of urinary bladder cancer have not been well explored in particular the memory CD4 T-cell and T helper follicular cell (Tfh). This study assessed the CD4 memory T-cells and (Tfh) cells infiltration by flowcytometry in tumor and autologous normal microenvironments of urinary bladder and subsequently their significance in antitumor immunology with their clinicopathological influences of urinary bladder cancer and a significant difference found in MTh cell and the Tfh cell infiltration between tumor and autologous normal microenvironment of urinary bladder in urinary bladder cancer pateints in addition to an important correlation between both MTh and Tfh cell in tumor microenvironment.
Keywords: T-helper follicular, CD4 memory T-cells bladder cance

1. Introduction

CD4 T cells become stimulated when primed by the Dendritic cells by presenting the antigens on class II MHC molecules, by the interaction with the T cell receptors (TCRs) on CD4 T-cells in association with CD3 molecules. The naïve T-cells priming can't be achieved by TCR signals alone, so activating DCs need to provide costimulatory signals. The activation of TCR with diminished costimulatory signaling resulting in T cell tolerance instead of CD4 memory T cells (Jenkins et al.,1987; Kearney et al.,1994; Stary et al.,2015).

Memory T cell (MT cells) generated as a result of T cell activation, but it depends on specific antigen type and amount, type of antigen presenting cell and inflammatory cytokines. Small amount of antigen, or diminished time of presentation, can be adequate for primary activation of T cell however this fails to produce memory cells (McLean-Tooke et al.,2008). On the other hand, excessively activated CD4 T cell cause highly differentiated cells which is not prone to be converted to the memory pool than lesser differentiated cells and chronic antigen exposure resulting into memory T cells to be exhausted which exhibit poor response upon reactivation (Crawford et al.,2014; Kahan et al.,2015,). At the same time memory cell formation can be affected by type of antigen since if the epitope liberated as a protein, instead of a sole peptide, enhance a wide range of T cell to pass into the memory pool (Baumgartner et al.,2012).

Memory T-cell have a vital role for both cellular and humeral immune responses. After T-cell expansion as a result of encountering an antigen , subsequent antigen clearance, and effector cells death, then after some of the remaining T-cells would be differentiated into memory T-cells (Siegrist, 2003). MT cells comprise subtypes of which found in the lymphoid organs called central MT-cells (TCM) that become greatly responsive upon reinfection, and the other subtype circulate through out various organs called effector MT-cells (Tem) (Pepper M et al.,2011 Sallusto et al.,1999). Another subtype of MT cells persist in peripheral tissues and do not recirculate called the tissue resident MT-Cells (TRM) (Jiang et al.,2012).The later cells express high motivation of activation so they play a critical role in defending epithelial tissues against infectious and inflammation in various tissue (Yang et al.,2007; Masopust et al.,2010;Gebhardt et al.,2009),these categories observed depend on expressing effector cytokine and cell surface of selectin and homing molecules (Jaigirdar and, MacLeod, 2015).

The generation of ideal CD4 T cell activation and later on memory T cells formation require targeting antigen to B cells. Sometimes primary CD4 T-cell reactions are weakened if the B cells do not exist; as well the pool of memory cells consistently faded (Linton et al.,2000; Whitmire et al.,2009). Since it has been found that B cells has an important role in CD4 memory T cell formation this persuaded to hypothesize the presence of a memory cells precursor subset of Tfh cells (Choi et al.,2013; Hale et al.,2013). As a result, CD4 T-cells encountering antigen presented by DCs primary Tfh cell differentiation would occur but sustained differentiation of Tfh cell require the presence of B cells (Poholek et al.,2010; Nurieva et al.,2008). Memory Tfh cell subset have a tendency to re-express the Tfh cell markers, after reactivation (Hale et al.,2013). Highly infiltrated breast cancer tumors with $CD4^+$ (Tfh) cells associated with extensive immune infiltrates, primarily in tertiary lymphoid structure germinal centers, suggesting the role of

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CD4⁺ Tfh cells as a predictor and prognostic factor for patient survival and treatment response (Gu-Trantien et al.,2013) .

T cell become progressively stimulated during cancer they became exhausted and loss their function; however, tumor growth control needs efficient T cell responses (Kamphorst et al.,2013).There is high correlation of T- cell immune responses and tumor progress which is revealed in lung ,breast, and ovarian cancer (Dieu-Nosjean et al.,2008;Hu et al.,2017; Zhang et al.,2003).The CD4⁺ memory T cell described to have a critical role in TME, as the colorectal cancer found to be highly infiltrated in comparison to normal tissue (Toh et al., 2021).The enrichment score of CD4⁺ memory T cell in breast cancer found to be greater in advanced stage of tumors (Deng et al.,2019).

The patient's response to tumor immunotherapy can be affected by TRM cells level. As in immune Checkpoint inhibitor therapy found to enhance the generation of (TRM) in melanoma-mice (Enamorado et al., 2017). When PD-1inhibitor therapy and (TCM) transfer boost (TRM) and conger tumor growth (Enamorado et al., 2017).

2. Materials and Methods

Patients and samples

Autologous tissues (normal and tumor) samples were taken from 17 patients undergoing tumor resection surgery for primary non-recurrent urinary bladder cancer at the Basra Teaching Hospital after written informed consent. Excluding patients with recurrent tumors or those who are on immune modulating therapy.

Fresh tumor and normal non-adjacent tissue were directly transferred into DMEM media then tissue specimen placed on ice while preparing for process of single cell sample generation.

Single cell formation process

Preparing 2X TTDR To make 2X TTDR ((Tumor-Tissue Dissociation Reagent) from BD Bioscience USA) solution:

Add 5 mL of DMEM to the amber vial containing TTDR.

Gently agitate periodically for 15 minutes at room temperature to ensure complete

reconstitution of the dried reagent.

Transfer the reconstituted TTDR to a labeled 50-mL conical centrifuge tube.

Discard amber vial.

Store at 4°C until needed

After we weigh tissue specimen the tissue minced and place into a fresh, labeled 100 × 20-mm glass petri dish containing 5 mL of 37°C DMEM. Mincing the tissue. Use two scalpels to mince the tissue to minute pieces in the petri dish. The resulting tissue pieces where as small as possible Approximately 30 minutes before tissue mincing has been completed, place the tube containing 2X TTDR (Tumor-Tissue Dissociation Reagent) from BD Biosciences (USA) in a 37°C water bath not longer than 30 minutes prior to use. Transfer contents of the petri dish (DMEM and tumor pieces) into conical tube containing 37°C 2X TTDR. The final volume in the conical tube should be 10 mL (5 mL warm TTDR +5 mL minced tumor in DMEM).

Digesting the minced tissue

We incubate the tubes containing the minced tissue and TTDR at 37°C for 30 minutes with mild but frequent agitation. After incubation, we add 25 mL of 1% BSA/DPBS/2 mM EDTA to the conical centrifuge tubes containing the dissociated tissue to bring total volume to 35 mL. Pass the contents of each tube through a fresh 70-µm cell strainer into a fresh, labeled conical tube. After washing the strainer with 10 ml DMEM media Then after Centrifuge the tubes at 250g for 8 minutes at room temperature.

Then we Removed the supernatant and resuspend the pellets in 2 mL of 1X BD Pharm Lyse™ solution from BD Bioscience, USA. Incubate for 15 minutes at room temperature. Add 40 mL 1% BSA/DPBS/2 mM EDTA. Centrifuge at 250g for 8 minutes at room temperature. Remove the supernatant and resuspend pellets in 2 mL DPBS/2 mM EDTA.

We use Leukocyte Activation Cocktail with Golgi Plug from BD Bioscience, (USA) for T-cell activation to secrete cytokines for assessment by immunofluorescent staining.

We thawed the cocktail at 37°C in a water bath

2 µL of the cocktail added for every 1 mL of the dissociated samples and mixed thoroughly.

Then after Placed in the incubator at 37°C with 5% CO2 for 6 hrs.

After activation the cells washed with FACS Staining Buffer to be used in immunofluorescent assessment. Then the cells washed with wash buffer and the cells resuspended in staining buffer, then after 50 µL of the resuspended cells added to each tube. Fluorochrome-conjugated monoclonal antibodies added (according to manufactured kit) then the mixture incubated in the dark for 15-20 min at 4°C.

The following fluorescent-labeled antibodies from BD (USA) were used: anti-CD4 PerCP-Cy5.5, Anti-Hu CD25 BB515, anti-CCR4 PE, anti-CD45RA PE-Cy7, anti-HU CD196 (CCR6) APC, anti-CD3 APC-H7, anti-Hu CD196 (CCR6) BV421, anti-HU CD127 BV421, anti-Hu CD161 BV510, anti- Hu CXCR5 BV510.

Tfh cells gating was (CD3+CD4+ CD25+ CD127+CD45RA-CXCR5+)

MTh cells gating was (CD3+CD4+ CD25+ CD127+CD45RA-CXCR5-).

3. Statistical Analysis

IBN SPSS statistics version 22 program used for analyzing our data, The data expressed as mean ± standard deviation. Wilcoxon Signed Ranks Test used for paired samples, while Kruskal Wallis Test to assess multiple types of samples. Spearman's Correlations test used to assess the presence of a correlation between CD4 MTh cell and Tfh cell infiltration in tumor a microenvironments of urinary

bladder cancer patients. P value was considered to be significant at ≤0.05.

4. Results

We found a considerably lower infiltration of CD4 MTh cells in urinary bladder cancer tumor microenvironment with mean percentage in CD4 (2.30±4.43) as TIL in comparison to normal urinary bladder tissue microenvironment mean percentage (10.84±11.63) the difference was statistically significant (p< 0.05) table (1).

The Tfh cell infiltration of urinary bladder tumor microenvironment mean percentage in CD4 T cell (4.85±9.56) was significantly lower than their infiltration in autologous normal tissue (8.16±8.80), (p<0.05) table (1).

We use of Spearman's Correlations to assess the presence of a correlation between CD4 MTh cell infiltration in autologous tumor and normal microenvironments of urinary bladder cancer patients showed no significant correlation (R=0.219, p>0.05), a statistical significant positive correlation found between the CD4 MTh cell infiltration and CD4 Tfh cell infiltration of urinary bladder tumor microenvironment (R=0.160, P<0.05) table(1) figure(2) .On the other hand there was no significant correlation between CD4 MTh and CD4 Tfh of normal urinary bladder microenvironment (R=0.274, P>0.05).

The mean percentage of CD4 MTh infiltration in normal urinary bladder microenvironment was higher in stage I (20.46±18.20) than that for stage II (9.84±11.95) and III (6.42±5.33) but it was not significant this might be due to low number of patients. On the other hand, CD4 MTh cell mean percentage in tumor microenvironment and Tfh cell in both normal and tumor microenvironment were statistically not different throughout disease stages table (3).

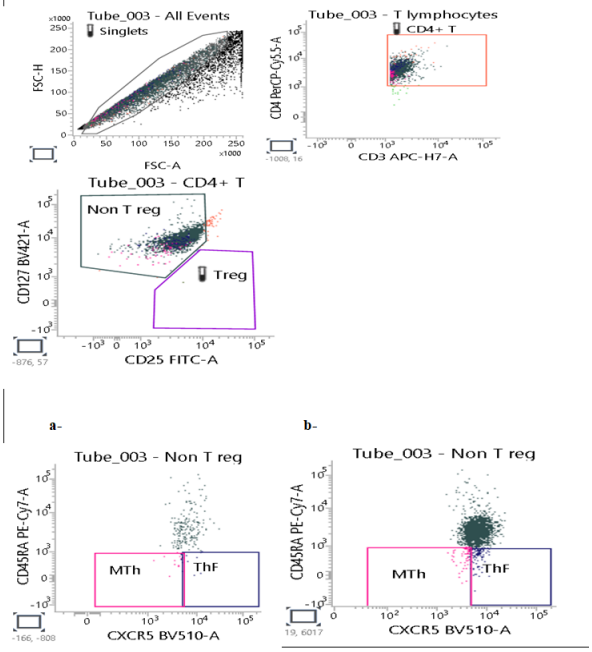

Figure 2: Shows the gating scheme for identification of MTh cells and Tfh cells

a-shows MTh and Tfh cells in normal microenvironment.
b-shows MTh and Tfh cells in tumor microenvironment.

Table 1: Comparison of prevalence of MTh and Tfh cells between normal and tumor microenvironment.				
	MTh normal	MTh tumor	Tfh normal	Tfh tumor
Mean±SD	10.84±11.63	2.30±4.43	8.16±8.80	4.85±9.56
Median	6.78	0.34	5.26	1.81
Min.-Max.	1.37-33.33	0.05-15.00	1.37-33.33	0.09-33.33
Wilcoxon Signed Ranks Test	0.021*		0.041*	
Level of significance if p<0.05				

Table 2: correlation between MTh and ThF cells in both normal and tumor microenvironment.				
		MTh Tumour	Tfh Normal	Tfh Tumour
MTh Normal	R	0.219	0.274	0.406
	Sig.	0.517	0.415	0.215
MTh Tumour	R		-0.455-	0.636*
	Sig.		0.160	*0.035
Tfh Normal	R			-0.055-
	Sig.			0.873
* Spearman's Correlations Correlation is significant at the 0.05 level.				

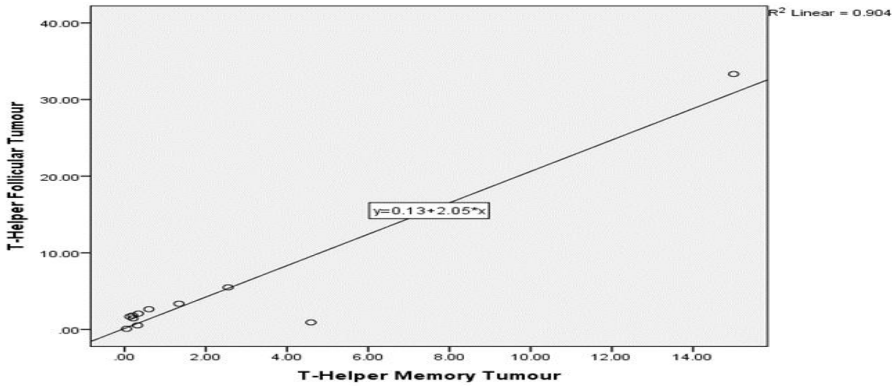

Figure 2: regression curve for correlation between MTh cells and Tfh cells tumor microenvironment

Table 3: MTh and Tfh cell infiltration of both normal and tumor microenvironment through stages of tumor.

Tumor Stage		MTh Normal	MTh Tumor	Tfh Normal	Tfh Tumor
1	Mean±SD	20.46±18.20	1.94±0.856	6.74±4.21	4.42±1.51
	Median	20.46	1.94	6.74	4.42
	Min.-Max.	7.59-33.33	1.34-2.55	3.77-9.72	3.36-5.49
2	Mean±SD	9.84±11.95	0.27±0.19	10.86±11.31	1.47±0.96
	Median	5.47	0.26	7.28	1.74
	Min.-Max.	1.37-33.33	0.05-0.60	2.94-33.33	0.09-2.64
3	Mean±SD	6.42±5.33	6.60±7.59	3.71±2.20	11.90±18.6
	Median	5.88	4.59	4.00	1.45
	Min.-Max.	1.37-12.00	0.21-15.00	1.37-5.75	0.92-33.33
Sig.*		0.424	0.091	0.469	0.227
* Kruskal Wallis Test,					

5. Discussion

The level of inflammation and lymphocyte tumor infiltration found to be correlated with patient response to therapy and overall survival (Pfannstiel et al.,2019). Studying Immune evasion in bladder cancer can add further insight about the therapeutic strategy to improve patient response. Immune evasion is the key feature of malignant cells, which encourage proliferation, survival, and dissemination. Thus, recognizing how tumor evade immune responses could boost the efficiency of anticancer therapies by eluding immunosuppression caused by tumor and enhancing the anti-tumor immune responses (Paul and Sergei et al.,2019). There is a critical association between T cell immune responses and tumor progress (Dieu-Nosjean et al.,2008, Hu et al.,2017, Zhang et al.,2003), CD4+ memory T cell found to be an important player in TME, higher infiltration of CD4 memory T cell observed in colorectal cancer tumor microenvironment than normal tissue (Ge et al.,2019); CD4+ memory T cell infiltration found to be associated with less advanced tumors (Pagès et al.,2005). In present study we tried to demonstrate the role of CD4 MTh cells in urinary bladder cancer microenvironment because of the limited studies about their role in tumor microenvironment and at least to my knowledge no study assess their role in bladder cancer microenvironment the except the Chinese by (Li et al., 2020), our results of CD4 MTh cell infiltration assessment in urinary bladder tumor and normal microenvironments by flow cytometry reveals extensively lower CD4 MTh cell infiltration of tumor than normal urinary bladder microenvironment , and the degree of CD4 MTh cell infiltration in tumor microenvironment was higher in stage I than II tumor , this become to some extent consistent with (Li et al., 2020) who found that in bladder cancer the CD4+ MTh cells had the lowest tumor infiltration in clinical stage IV, and the bladder cancer patients with high infiltration of CD4+ MTh cells might have a better clinical prognosis (Li et al., 2020). As the antigen cognate CD4+ MTh cells boost up the antigen cognate CD8+ memory T cells expansion and infiltration in addition to the accumulation of these cells in tumor microenvironment .CD4+ T-cell memory T cells substantially related to empowering antitumor CD8+ T-cell immune responses (Lai et al.,2011). As we found these cells diminished in tumor microenvironment of urinary bladder cancer patients so as the antitumor cytotoxic CD8 T cell responses would be extremely weak.

Since it has been found that B cells has an important role in CD4 memory T cell formation this persuaded to hypothesize the presence of a memory cells precursor subset of Tfh cells (Hale et al.,2013; Choi et al.,2013). As a result, CD4 T-cells encountering antigen presented by DCs primary Tfh cell differentiation would occur but sustained differentiation of Tfh cell require the presence of B cells (Nurieva et al.,2008; Poholek et al.,2010). In the present study we found there is an extremely lower Tfh cell infiltration of urinary bladder tumor microenvironment in comparison to normal microenvironment, this reflects a state of immune suppressed tumor microenvironment, while (Wu et al.,2020) found no significant difference of Tfh and CD4 memory cell infiltration between normal and tumor microenvironment. differences in Tfh cell associations in more than 100 types of cancer depending on the cancer (Shalapour et al.,2017). Highly infiltrated breast cancer tumors with (Tfh) cells associated with extensive immune infiltrates, primarily in tertiary lymphoid structure germinal centers, refers to $CD4^+$ Tfh cells role as a predictor and prognostic factor for patient survival and treatment response (Gu-Trantien et al.,2013). In the same context the present study shows a significant positive correlation between Tfh cell and CD4 memory T cells infiltration in tumor microenvironment, this correlation may explained by recent studies revealed that Tfh cells participate in memory pool of CD4 T cells (Yu et al.,2012, Friedman et al.,2010, Zeng et al.,2019), since Tfh cells participate in memory CD4 T cell formation, indicating that the Tfh cell and memory CD4 T cell both sharing pathways of development (Ning et al.,2021). In the same context Bcl6 (Tfh transcription factor) has been associated with memory T cell development, especially development of memory CD8 T cell (Crotty et al.,2010, Rutishauser et al.,2009), and as Tfh cell affect CD4 MTh cell generation , infiltration and subsequent CD4 T cell help for cellular and humoral immune responses in tumor microenvironment this may refer to the vital

role of Tfh cell in tumor immunopathogenesis, so it might be enrolled as a predictor and prognostic indicator for patients treatment response and survival.

The tumor antigens presented by tumor-specific B cells which confer signals that are essential for IL-21 yielding Tfh cells differentiation. in turn, IL-21 augment the performance of effector CD8+ T cells augmented by IL-21, resulting in suppression of tumor growth. However, not all the tumor neoantigens have the ability to activate B cell, Tfh cell and IL21 secretion pathways, for instance the tissue specific CD4 T cells and B cells have to recognize neoantigens of the tumor. The tumor-specific Tfh cell-B cells interactions at the germinal center were essential for tumor control. If the T cell interactions with B cell or the IL-21 receptor were reduced. The effector CD8 T cell found to be declined. The IL-21 principally yielded by Tfh cells, that essentially needs the B cells for development. Furthermore, the tumor-specific Tfh cell immune reactions were dependent on B-cell-recognized neoantigens expressed by tumors. Consequently, the tumor-specific CD4+ T cells fate might be controlled by the tumor-neoantigens themselves permitting them to be engaged with tumor-specific B cells. (Cui et al.,2020).

Since Whitmire JK et al.,2009 found the absence of the B cells was correlated with Tfh cells absence as well , so according to the earlier observations gives us a clue about the vital role of B cell in memory CD4 T cell development, this gives us an indicator about relationship between B cell, Bcl6, Tfh cells, and CD4 MTh cell and subsequently the infiltration of CD8 T cell in the TME and subsequent tumor growth regression, for decades there was a concentration about the role of cytotoxic immune responses in tumor growth regression , this study observed the diminished infiltration of both CD4 MTh cells and the Tfh cells in urinary bladder tumor microenvironment in comparison to the autologous normal microenvironment with the positive correlation between them this link confirm the role of Tfh cells based immune therapy can be promising in enhancing response to immunotherapy .

Immune checkpoint receptors high expression like PD-1 and TIM-3 in tissue resident memory T cells within lung cancer patients (Djenidi et al.,2015), same results found in normal tissues (Mackay et al.,2013).at the same context this established in other cancers as well (Djenidi et al.,2015, Ganesan et al.,2017, Nizard et al.,2017). There was a strong association between expression of tissue resident MTh cells and exhaustion molecules like PD-1, Tim-3, TIGIT and LAG-3 (Komdeur et al.,2017). The cytolytic mechanisms found to be empowered by the blockade of PD-1 on the tissue resident MTh cells (Djenidi et al.,2015). The induction of the circulating Th cells as a result of immune checkpoints inhibitor therapy in the same context result in enhanced B cell activation capacity (Sánchez-Alonso et al.,2020). This suggest the important role of the CD4 MTh cells and the Tfh cells for induction of antitumor immune responses , and The important relationship between CD4 MTh and Tfh cell infiltration would makes them as important target of immune therapy through augmenting these cell infiltration and function in tumor microenvironment at the same time the generation of the Tfh cells (which needed for CD4 MTh formation) needs the tumor neoantigens to be recognized by the cognate B cells and subsequent Tfh-B cells interaction so this may refers to B cell failure to recognize tumor neoantigen.

Tumors express diverse anti-apoptotic and immunosuppressive factors like IL-10, and IL-6 resulting in a highly immune suppressive microenvironment. At the same time the tumor microenvironment contains immune cells with immunoinhibitory activity such as regulatory T cells and tumor-associated macrophages. These all have been illustrated in bladder cancer, that had an extremely immunosuppressive microenvironment which characterized by high expression of the PD-L1 (PD-1 ligand) (Crispen and Kusmartsev et al.,2020). Many tumors of murine or human tumor cell lines didn't express PD-L1, however, on the other hand, large number of tumors exhibit high PD-1 expression (Dong et al.,2002, Prima et al.,2017). This may reflect the role of the immune and inflammatory cells recruited to the tumor microenvironment, in augmentation of PD-L1 expression.

The high expression of checkpoint receptors on tissue resident memory CD4 T cell, and their critical in situ presence in tumor microenvironment and their proliferation in situ in response to regional stimulus. Also tissue resident memory CD4 T cells rich microenvironment found to be augmented in cognate CD8+ T cells, this mark them as a potential effector of anti-PD-1 immunotherapy, at the same time their induction by the means of cancer vaccines or other immune therapy modes may be vital for the response to immunotherapy.

6. Conclusion

Urinary bladder cancer has a very immunosuppressed microenvironment, in which the CD4 MTh and the Tfh cell act as important indicators for prediction of patient's response to therapy and survival. The Tfh cell and the CD4 T cell augmentation in urinary bladder cancer microenvironment can act as an important participant in boosting response to immune therapy since they enhance antitumor cellular and humoral immune responses and augments antitumor cytolytic activities.

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