Decidual infiltration of FoxP3+ regulatory T cells, CD3+ T cells, CD56+ decidual natural killer cells and Ki-67 trophoblast cells in hydatidiform mole compared to normal and ectopic pregnancies

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Abstract. Hydatidiform moles are considered pre-cancerous lesions of gestational trophoblastic neoplasia and are associated with an aberrant immune response. This preliminary study aimed to evaluate the feasibility of measuring the presence of immune cells as potential prognostic markers for hydatidiform moles. Immunohistochemical staining of FoxP3+ regulatory T cells, CD3+ T cells, CD56+ decidual natural killer cells and Ki-67+ trophoblast cells was performed on 32 samples. Samples were from complete hydatidiform moles, partial hydatidiform moles, ectopic pregnancies, gestational age-matched normal elective pregnancy terminations (normal pregnancies) and gestational trophoblastic neoplasias. FoxP3+ regulatory T-cell infiltration was highest in the complete hydatidiform moles and lowest in the normal pregnancy samples. The normal pregnancy cases showed significantly fewer FoxP3+ regulatory T cells compared to the ectopic pregnancy cases (p=0.037) and compared to the combination of all of the other groups (p=0.044). Normal pregnancy samples also showed the lowest infiltration of CD3+ T cells and the highest number of CD56+ decidual natural killer cells; conversely, gestational trophoblastic neoplasias showed the highest infiltration of CD3+ T cells and the lowest number of CD56+ decidual natural killer cells. The numbers of Ki-67+ trophoblast cells were highest in the gestational trophoblastic neoplasias (688/1,000 trophoblast cells) and lowest in the partial moles (87/1,000 trophoblast cells). Our results suggest that regulatory T cells may be involved in the progression of complete hydatidiform moles. A larger cohort study is required to assess whether immune cells are effective prognostic markers in gestational trophoblastic diseases.

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Introduction

Hydatidiform moles (HM) or molar pregnancy is a unique entity among gestational trophoblastic diseases (GTDs), characterized by abnormal proliferation of placental trophoblasts accompanied by an excess of paternal genes. This pathological form of pregnancy persists until the 1st trimester before clinical symptoms become evident (1-4). The incidence of HM is highest in Asian countries and comparatively low in Western countries (5,6). HM appears as complete moles (CM) or partial moles (PM), based on histopathological features and karyotypes that confer the potential for local invasion and widespread metastasis. The level of aggressiveness varies according to mole type; they may lead to persistent disease or post-molar gestational trophoblastic neoplasia (GTN). Thus, HMs are considered the pre-cancerous lesions of GTN (3,7).

Most CMs are cytogenetically diploid with a 46XX karyotype. Both sets of chromosomes are of paternal origin, since the CM results from fertilization of an empty ovum by a haploid (23X) spermatozoa; alternatively, dispermic CMs develop from an empty ovum fertilized by two haploid (23X and/or 23Y) spermatozoa. Both types of CMs are totally paternally derived and represent a complete intrauterine allograft in the mother. In contrast to CMs, PMs are generally triploid; PMs retain maternal chromosomes, but also have an excess set of paternal chromosomes. PMs result from fertilization of a normal haploid ovum (23X) by two haploid spermatozoa (3,4,7). In CMs, the complete allograft conceptus is expected to provoke a maternal immune response, with immune cell invasion leading to rejection. PMs may also provoke an altered immune response compared to normal pregnancy (NP), due to the excess paternal genetic material.

Ectopic pregnancy (EP) is another type of first trimester abnormal pregnancy. It is the most common early pregnancy

complication, representing 1.5-2.0% of pregnancies in Western countries. EP is a fertilized ovum that is implanted outside the endometrial cavity (over 80% of EPs occur in the fallopian tube); this may also lead to an aberrant immune response compared to NP (8,9).

Acceptance of the fetus in a NP, which expresses paternally inherited alloantigens, by the mother during pregnancy is a unique example of how the immune system reshapes a destructive alloimmune response into a state of tolerance. Many mechanisms protect the fetus from the maternal immune system. These mechanisms include complex interactions between immune cells and trophoblast cells. Previous studies have shown that $CD56^{+(bright)}CD16^{-(dim)}$ decidual natural killer (dNK) cells and $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs) are important for establishing a successful pregnancy (10). Since HM is a pregnancy-based tumor, it is worth investigating how the presence of this pregnancy-related subpopulation of T cells, together with classical T cells ($CD3^{+}$ T cells), are involved in tumor immunity.

During the first trimester of a NP, dNK cells comprise the majority of decidual lymphocytes and are considered to be a special subset of NK cells. The dNK cells have less cytotoxic activity than the $CD56^{dim}CD16^{bright}$ peripheral NKs, which are specialized in killing virally infected and transformed cells. Instead of triggering cytotoxicity, the dNK cells interact with non-classical human leukocyte antigens (HLA) expressed by trophoblast cells, particularly HLA-E and HLA-G. This interaction stimulates the dNK cells to secrete cytokines and angiogenic factors, which are essential for trophoblast invasion and vasculature modifications that nourish the conception product (10-17).

In contrast to the NK cell population, which participates in innate immunity, Tregs participate in the adaptive immune system. Tregs are a distinct subpopulation of T cells that exert a negative regulatory effect on the immune response in a cell contact-dependent manner. Tregs may play a central role in eliminating autoreactive T cells, which is necessary in the course of a normal immune response in order to prevent autoimmune disease (10,18-20). However, the presence of Tregs leads to alterations in the specificity of tumor immunity, which may contribute to tumor growth and progression. This is substantiated by the fact that a high number of infiltrating Tregs has been proven to be a poor prognostic factor in breast, ovarian, cervical and other types of cancer (21-23). In addition, significant numbers of infiltrating $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs have been observed in the decidual tissues of HMs (24).

This pilot study aimed to compare the levels of $FoxP3^{+}$ Tregs in the decidual tissues of CMs and PMs to those observed in NPs and EPs (tubal). In addition, we investigated whether there were significant differences in the proliferation status of trophoblasts between the groups studied. Our ultimate goal was to evaluate the feasibility of these measurements in predicting persistent disease and post-molar GTN.

Materials and methods

Materials. H&E slides were retrieved from the archives of the Department of Pathology, Leiden University Medical Center (LUMC), Leiden, The Netherlands. H&E slides of GTDs from 1965 to 2009 were retrieved. Evaluation of H&E-stained slides was performed with standard light microscopy. Samples were selected based on gestational age (≤1st trimester) and the presence of decidual tissue, except for the CM, choriocarcinoma (CCA) and invasive mole (IM) cases, which were selected as the number of cases was limited. Then, from the same archive, H&E-stained, matched paraffin-embedded tissue blocks were retrieved with the following histopathological diagnoses: CM (5 cases, mean age 24.9 years, range 20-47), PM (12 cases, mean age 29.7 years, range 24-36), CCA (2 cases), gestational age-matched normal elective pregnancy termination/social indicative abortions (NP; 8 cases, mean age 24.9 years, range 15-31), IM (1 case) and EP (4 cases, mean age 37.5 years, range 29-41). These cases were divided into five groups according to the diagnosis, while IM and CCA were combined to form a GTN group (mean age 34.8 years, range 30-41).

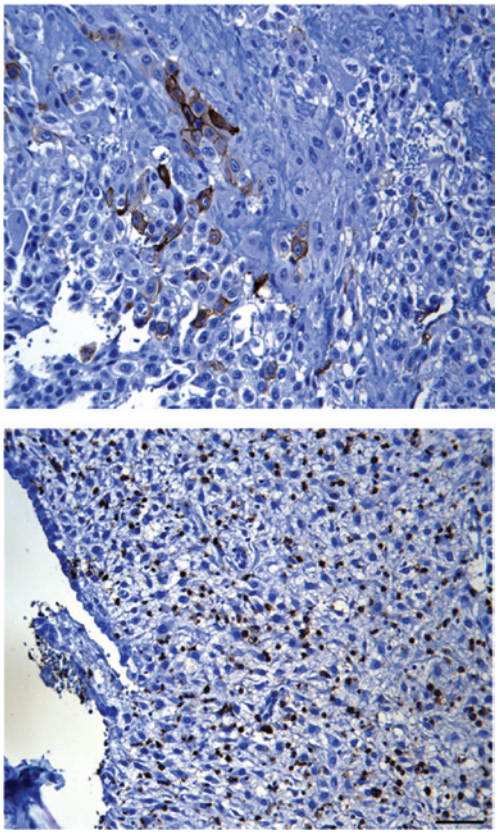

Figure 1. Light microscope immunohistochemical images of (A) non-classical $CD56^{+}$ cells in decidual tissue of an ectopic pregnancy; (B) infiltration of $CD56^{+}$ cells in decidual tissue from a normal pregnancy. Magnification, x400; scale bar, 120 μm.

Immunohistochemical staining. Immunohistochemical staining was performed for the identification of $FoxP3^{+}$ Tregs, $CD56^{+}$ dNK cells, $CD3^{+}$ T cells and $Ki\text{-}67^{+}$ cells (proliferation marker) in the tissue samples. Briefly, 4-μm paraffin sections were cut on APES-coated slides and dried at 37˚C overnight. After deparaffinization, endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol. Sections were then rehydrated sequentially in 70 and 50% ethanol. Heat-induced antigen retrieval was performed by immersing the slides in 10 mM Tris-HCl, 1 mM EDTA, pH 9.0, and heating in a microwave oven for 10 min. After washing twice with distilled water and phosphate-buffered saline (PBS), the sections were

Table I. Detection of FoxP3+, CD56+ and CD3+ cells in the decidua of normal and genetically aberrant pregnancies; and Ki-67+ trophoblast cells.

	CM (n=5) (cell count)	PM (n=12) (cell count)	NP (n=8) (cell count)	GTN (n=3) (cell count)	EP (n=4) (cell count)
FoxP3+ Tregs					
Mean ± SD	7.11±7.11	3.35±3.19	0.9±0.86	2.41±2.12	3.46±2.65
Median	7.24	2.96	0.99	3.29	2.63
Range	0-17.77	0-10.53	0-19.97	0-3.95	1.32-7.24
CD56+ dNK					
Mean ± SD	75.02±51.58	82.23±60.45	102.88±48.9	0.36±0.62	34.65±29.31
Median	69.03	80.54	106.49	0	33.18
Range	20.87-129.50	0-239.74	29.43-194.79	0-1.07	1.07-71.17
CD3+ T cells					
Mean ± SD	106.2±63.39	95.71±63.57	52.81±36.83	356.97±60.03	78.47±46.70
Median	128.97	84.49	42.44	356.97	90.15
Range	29.61-166.47	37.51-257.28	7.90-113.18	314.52-399.41	12.50-121.07
Ki-67+ trophoblastS					
Mean ± SD	287.75±219.52	87.42±86.92	258.75±131.38	688±356.80	290.75±217.26
Median	307.5	62.5	230	894	321.5
Range	13-523	0-204	154-575	276-894	3-517

CM, complete hydatidiform mole; PM, partial hydatidiform mole; NP, normal pregnancy; GTN, gestational trophoblastic neoplasia; EP, ectopic pregnancy; dNK, decidual natural killer cells.

incubated overnight with primary antibodies diluted in PBS with 1% bovine serum albumin. The primary antibodies were monoclonal mouse anti-human FoxP3 antibody (1:100 μl; 236A/E7; Abcam), monoclonal mouse anti-human CD56 (1:200 μl; 123C3; Dako), monoclonal mouse anti-human CD3 (1:300 μl; F7.2.38; Dako) and monoclonal mouse anti-human Ki-67 antigen (1:200 μl; MIB-1; Dako). The Powervision kit from the immunologic and standard diaminobenzidine reaction was used to visualize the antibody staining.

Quantification. We randomly selected ten medium power fields (magnification, x250) to count FoxP3+ Treg and CD3+ T cells. The quantification of CD56+ dNK cells was performed by selecting five random high power fields (magnification, x400). The assessment of trophoblast proliferation was performed per 1,000 cells in a high power field (magnification, x400).

Statistical analysis. Differences between groups were determined using the Mann-Whitney U test. Significance was determined as $p<0.05$. All statistical analyses were performed with SPSS 17.0. Additionally, ratios of immune cells were calculated as follows: FoxP3+ Tregs/CD3+ T cells, FoxP3+ Tregs/CD56+ dNK cells, FoxP3+ Tregs/Ki-67+ trophoblast cells, CD3+ T cells/CD56+ dNK cells, CD3+ T cells/Ki-67+ trophoblast cells and CD56+ dNK cells/Ki-67+ trophoblast cells.

Results

Immune cell infiltration. Immune cell infiltration was observed in nearly all of the cases. FoxP3+ Treg infiltration was not observed in 1 CCA case (1/2), 1 CM case (1/5), 3 PM cases (3/12) and 3 NP cases (3/8). FoxP3+ Tregs infiltrated the villi in 1 PM case (1/12) and 1 NP case (1/8). CD56+ dNK cells were present in all cases, except 2 CCA and 1 IM case (3/3 GTN cases). In addition, membranous CD56 positivity was observed in large cells in 2 EP cases (2/4; Fig. 1A). These cells had large cytoplasmic areas that did not appear in the classical NK cells observed in NP (Fig. 1B). CD3+ T cells were found in all of the cases, but Ki-67 staining was negative in 1 CM case (1/5) and 1 PM case (1/12). In addition, Ki-67 positivity was found in <15/1,000 trophoblast cells in 1 CM case (1/5), 1 EP case (1/4) and 4 PM cases (4/12). The mean counts and the decidual infiltration of FoxP3+, CD56+, CD3+ and Ki-67+ trophoblast cells are shown in Table I and Figs. 2-4.

FoxP3+ regulatory T cells. The NP group showed significantly lower numbers of infiltrating FoxP3+ cells compared to the EP group ($p=0.037$) and compared to the combined CM, PM and GTN groups ($p=0.044$). The number of FoxP3+ Treg cells was highest in CM (Fig. 5); however, the differences among groups were not significant, possibly due to the limited sample sizes of GTDs.

CD56+ decidual natural killer cells. The number of CD56+ dNK cells was highest in the NP group and lowest in the GTN group. The NP group showed significantly higher CD56+ dNK cell infiltration than the EP ($p=0.027$) and GTN ($p=0.014$) groups. Higher infiltration was also observed in the EP ($p=0.048$), PM ($p=0.02$) and CM ($p=0.024$) groups compared to the GTN group.

CD3+ T cells. The number of CD3+ T cells was highest in the GTN group (Fig. 6). The numbers of CD3+ T cells were signifi-

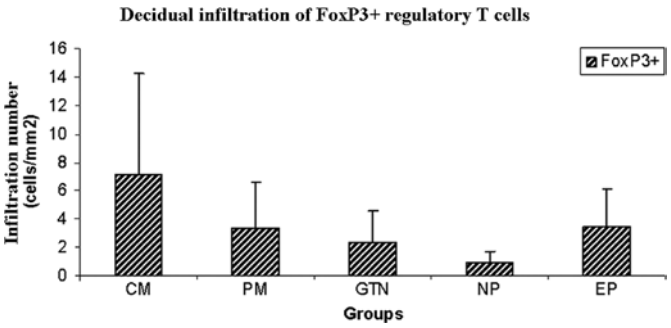

Figure 2. Decidual infiltration of FoxP3$^+$ regulatory T cells in decidual tissues of normal and aberrant pregnancies. CM, complete hydatidiform moles (n=5); PM, partial hydatidiform moles (n=12); GTN, gestational trophoblastic neoplasias (n=3); NP, normal pregnancies (n=8); EP, ectopic pregnancies (n=4).

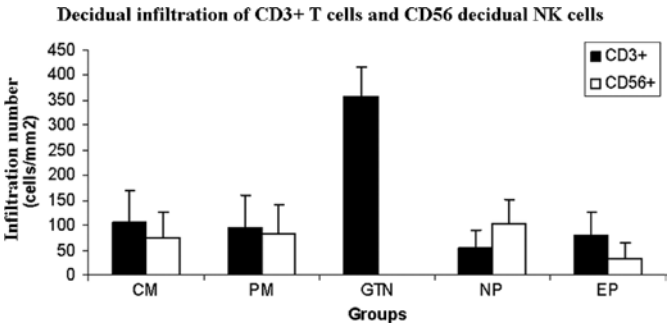

Figure 3. Decidual infiltration of CD3$^+$ T and CD56$^+$ decidual natural killer cells in decidual tissues of normal and aberrant pregnancies. CM, complete hydatidiform moles (n=5); PM, partial hydatidiform moles (n=12); GTN, gestational trophoblastic neoplasias (n=3); NP, normal pregnancies (n=8); EP, ectopic pregnancies (n=4).

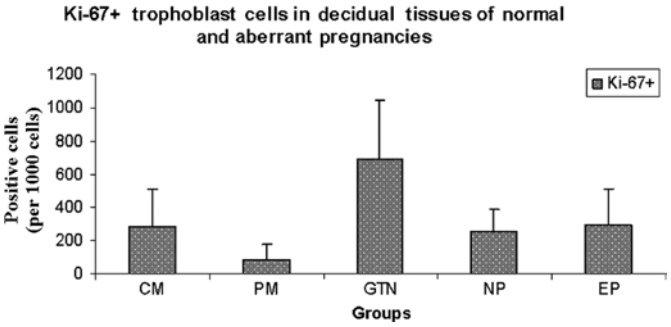

Figure 4. Ki-67$^+$ trophoblast cells in decidual tissues of normal and aberrant pregnancies. CM, complete hydatidiform moles (n=5); PM, partial hydatidiform moles (n=12); GTN, gestational trophoblastic neoplasias (n=3); NP, normal pregnancies (n=8); EP, ectopic pregnancies (n=4).

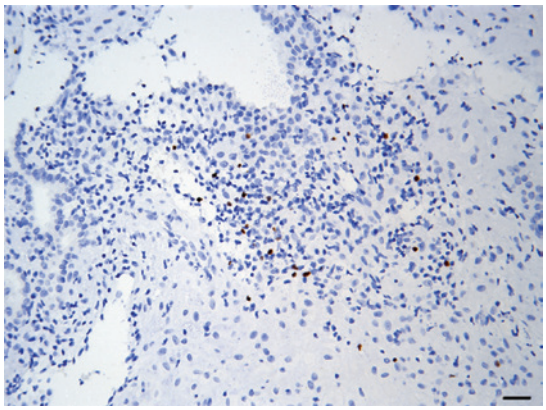

Figure 5. Infiltration of FoxP3$^+$ cells in decidual tissues of complete hydatidiform moles. Magnification, x250; scale bar, 100 μm.

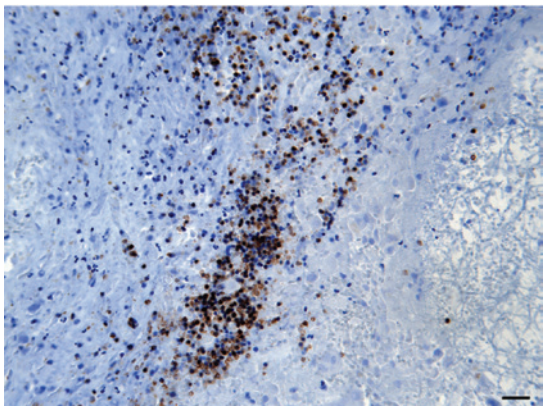

Figure 6. Infiltration of CD3$^+$ cells in decidual tissues of choriocarcinoma. Magnification, x250; scale bar, 100 μm.

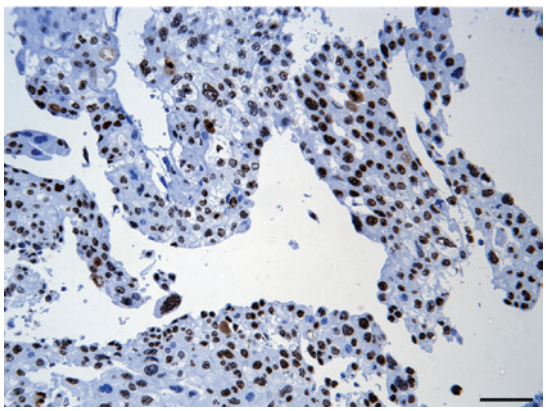

Figure 7. Ki-67$^+$ trophoblast cells in choriocarcinoma. Magnification, x400; scale bar, 120 μm.

cantly lower in the NP (p=0.037) and PM (p=0.030) groups compared to the GTN group (Fig. 3). The number of CD3$^+$ T cells was also significantly lower in the NP group compared to the PM group (p=0.048).

Ki-67$^+$ trophoblast cells. Ki-67 trophoblast cell positivity was highest in the GTN group (Figs. 4 and 7). The NP and PM groups were significantly lower than the GTN group (p=0.024 and p=0.009, respectively), and the NP group was significantly different than the PM group (p=0.02). The number of Ki-67$^+$ trophoblast cells was significantly lower in the PM compared to the CM group (p=0.039). Notably, Ki-67$^+$ positivity was observed in both trophoblast cells and in cells within the decidua.

Cell ratios. Ratios between immune cells and Ki-67$^+$ trophoblast cells were also calculated. The mean cell/cell ratios are shown in Table II. The FoxP3$^+$ Treg/CD56$^+$ dNK cell ratio was significantly different between the NP and EP groups

Table II. Ratios of T cells to natural killer cells and immune cells to proliferative cells in the decidua of normal and genetically aberrant pregnancies.

	CM (cell count)	PM (cell count)	NP (cell count)	GTN (cell count)	EP (cell count)
FoxP3+ Treg/CD56+ dNK					
Mean ± SD	0.169±0.182	0.057±0.056	0.015±0.022	3.692 ± na	0.403±0.565
Median	0.137	0.047	0.009	3.692	0.165
Range	0-0.44	0-0.18	0-0.07	3.69-3.69	0.05-1.23
CD3+ T cell/CD56+ dNK					
Mean ± SD	3.030±3.459	1.299±1.095	0.795±1.123	373.280 ± na	4.989±4.699
Median	0.996	0.954	0.482	373.280	3.554
Range	0.38-7.98	0.52-4.41	0.07-3.53	373.28-373.28	1.16-11.68
CD3+ T cell/Ki-67+ trophoblasts					
Mean ± SD	2.696±4.825	15.285±34.984	0.231±0.165	0.793±0.490	10.238±20.080
Median	0.394	1.693	0.211	0.793	0.2845
Range	0.08-9.92	0.27-113.83	0.04-0.48	0.45-1.14	0.02-40.36
CD56+ dNK/Ki-67+ trophoblasts					
Mean ± SD	2.628±4.889	14.820±35.151	0.460±0.279	0.0004±0.0007	2.235±4.220
Median	0.210	1.275	0.392	0	0.187
Range	0.13-9.96	0-119.33	0.13-0.85	0-0	0-8.56

CM, complete hydatidiform mole; PM, partial hydatidiform mole; NP, normal pregnancy; GTN, gestational trophoblastic neoplasia; EP, ectopic pregnancy; dNK, decidual natural killer cells.

(p=0.017). The CD3+ T cell/CD56+ dNK cell ratio was significantly different between the NP and EP groups (p=0.017), between the NP and PM groups (p=0.026), and between the EP and PM groups (p=0.037). The CD3+ T cell/Ki-67+ trophoblast cell ratio was significantly different between the NP and PM groups (p=0.001). The CD56+ dNK cell/Ki-67+ trophoblast cell ratio was significantly different between the GTN and the NP (p=0.014), the EP (p=0.032), the PM (p=0.023) and the CM (p=0.032) groups.

Discussion

Although several studies have previously investigated the presence of immune cells in HM and CCA cases (7,24-26), this was the first study to assess whether these particular immune and proliferative cells together may be useful markers for these cases.

We found that the number of FoxP3+ Tregs decreased in the order: CM > EP > PM > GTN > NP. Nevertheless, the number of FoxP3+ Tregs was only significantly different between the NP and EP groups, and between the NP and all the GTDs combined (CM + PM + GTN).

We found a moderate variation in the FoxP3+ count within the study group. This may have been caused by the sampling process obtained from the curettage. Since FoxP3+ Tregs work mainly in a cell contact-dependent manner, they are expected to be found on the implantation site area, but such locations are relatively hard to find from the curettage samples, therefore all counts were performed on the feto-maternal interface or decidual area.

It has been reported that the numbers of decidual and/or peripheral blood CD4+CD25+ Tregs increase during early pregnancy. This may provide the maternal immune system with tolerance to the presence of paternal antigens during pregnancy, as CD4+CD25+ Tregs are involved in the regulation of immune responses and peripheral tolerance. Moreover, several *in vitro* studies have demonstrated that CD4+CD25+ Tregs suppress proliferation and IFN-γ production by effector T cells. This stimulates the expression and activation of indoleamine 2,3-dioxygenase, a catabolic enzyme involved in tryptophan degradation. Reduced tryptophan concentrations in the culture medium was reported to be associated with decreased activation of T cells and T-cell deletion (10,19,20,26).

A recent study demonstrated that FoxP3+ Tregs are attracted to the feto-maternal interface by the chemoattractant properties of human chorionic gonadotropin hormone (hCG) in CCA cell lines. Their results suggest that blastocysts, and later trophoblast cells, secrete hCG to attract Tregs to the feto-maternal interface in order to orchestrate immune tolerance towards the conception product. Low expression of hCG was shown to correspond to low expression of FoxP3, which is critical for suppressor T-cell activity (27,28).

This may not fully explain our finding that FoxP3+ Treg infiltration was low in the GTN group. However, our result was based on a very limited GTN sample size. In another study, FoxP3+ Treg infiltration was significantly higher at the implantation site of HM (CM and PM) compared to the implantation site in NP, but they did not perform comparisons to CCA cases (7).

Our results also showed that CD56+ dNK cell infiltration was highest in the NP group and lowest in the GTN group. This was consistent with one study that reported a low number of CD56+ dNK cells in spontaneous abortions, which may have

been due to abnormal chromosomes. Moreover, others have reported a significantly increased percentage of $CD56^+$ dNK cells in missed abortions. In a study of gestational trophoblastic diseases, the number of $CD56^+$ dNK cells was reported to be significantly decreased in HM and CCA cases (25).

In contrast to Treg and NK cells, the total number of T cells was highest in the GTN group and decreased in the order: GTN > CM > PM > EP > NP. It may be postulated that the high number of $CD3^+$ T cells in the GTN, CM and PM groups was due to increased recruitment, local proliferation or increased turnover rates of these T cells in response to the molar trophoblasts (7). Another study that investigated the presence of $CD3^+$ T cells in gestational trophoblastic diseases reported that the number of $CD3^+$ T cells was high in HM and CCA cases, although it was not mentioned which type of HM was included in the study (24,25).

Significant results were obtained from the $FoxP3^+$ Tregs/$CD56^+$ dNK and $CD3^+$ T cells/$CD56^+$ dNK ratios; the highest ratio was found in the GTN and the lowest in the NP cases. The $FoxP3^+$ Tregs/$CD56^+$ dNK ratio may represent an immune-modulator factor, since both cells play important roles in host tolerance; however, additional studies are required to draw a firm conclusion regarding this matter, since for some samples the counts of $CD56^+$ dNK cells were nil and unable to be calculated. The relationship between these two cells is yet to be determined. The $CD3^+$ T/$CD56^+$ dNK ratio may correspond to the antitumor response of the host to pro-tumor immune cells and the $CD56^+$ dNK infiltration may explain these results.

In addition to the various immune cell subpopulations in NP and GTN, we studied the expression of the Ki-67 cell proliferation marker. The usefulness of Ki-67 staining for differentiation of gestational trophoblastic diseases is controversial (29,30). Here, we showed that there was no significant difference between NP and PM or NP and CM cases. The GTN was the only group found to be significantly different from the NP group, based on the proliferation of trophoblast cells, with an average of 688 Ki-67-positive cells per 1,000 trophoblasts. We also observed Ki-67 positivity in the decidua. The Ki-67-positive cells included large stromal-decidual cells and small cells with large nuclei that appeared to be immune cells. One study found that $CD56^+$ NK cells expanded and became Ki-67^+ in IL-2-supplemented mixed lymphocyte cultures, depending on the lack of ligand for killer inhibitory receptors on the stimulator cells (31). To make a firm conclusion on the applicability of Ki-67 for differential diagnoses between PM and CM, more cases should be examined.

In conclusion, our results indicated that there was an actual difference in decidual immune cells among the study groups investigated; however, due to the limited sample sizes, additional studies are warranted to confirm our results.

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