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Characterization of the Immune Cell Repertoire in the Normal Fallopian Tube

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Abstract

Recent studies implicating the fallopian tube as the site of putative precursors of ovarian serous carcinoma and the hypothesis that injury, inflammation and repair of the ovarian surface epithelium at the time of ovulation may be contributing factors to ovarian carcinogenesis, prompted us to undertake a comprehensive analysis of the immune cells in the normal fallopian tube. Accordingly, the aim of this study was to provide a base line for future studies exploring the relationship of inflammation to the early events of ovarian carcinogenesis by characterizing the immune cell repertoire in thirteen normal human fallopian tube, combining digital microscopy of immunostained slides and flow cytometry of fresh single cell suspensions, with a panel of markers that identify the most important adaptive and innate immune cells. We found that $CD45^+$ leucocytes are regularly observed in the fallopian tube and are mainly composed of $CD163^+$ macrophages, $CD11c^+$ dendritic cells and $CD8^+$ T-cells. In addition, there are minor populations of $CD56^+$ NK cells, $CD4^+$ T-cells, $CD20^+$ B-cells, $TCR\gamma\delta^+$ T-cells, and, among dendritic cells, $CD207(Langerin)^+$ Langerhans cells. The cellular mapping that we performed indicates that the local immune system in the human fallopian tube is composed of a mixture of innate and adaptive immune cells, many of which are recognized as playing a role in cancer immune surveillance. This local immune system could provide a first line of defense against early precancerous lesions and could potentially be exploited for immune-based therapies.

Keywords

fallopian tube; immune system; intraepithelial lymphocytes; dendritic and NK cells; digital microscopy

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Disclosure/Conflict of Interest

The authors declare absence of a conflict of interest.

INTRODUCTION

In addition to its critical role in fertilization[1], the fallopian tube, acts as a conduit for a variety of pathogens entering the pelvis. It, therefore, contains populations of immune cells that provide surveillance against these pathogens[2] and previous studies have shown that both the innate and the adaptive arms of the immune system are involved. Thus, attention has been directed to the immunology of the fallopian tube, in so far as infection[3] and tolerance towards the fetus and pregnancy-related diseases[4] are concerned, but the details of the immune system in this location remain poorly understood. Recent studies have implicated the fallopian tube as the site of origin of ovarian low-grade[5–6] and high-grade serous carcinoma[7–10]. These observations as well as studies showing that the density and composition of the immune cell infiltrate in human ovarian cancer, including T- and B-cells, NK-cells and macrophages, have prognostic significance in predicting response to chemotherapy[11–14], prompted us to undertake a comprehensive analysis of the immune cells in the fallopian tube as we reasoned that the immune system could potentially play a role in cancer immune surveillance. Accordingly, the present study was undertaken to characterize the immune cell repertoire of the normal human fallopian tube using immunohistochemistry (IHC) and flow cytometry, with a panel of markers that identify the most important adaptive and innate immune cells. The findings in this study lay the groundwork for subsequent studies of the various immune cell populations in potential ovarian cancer precursor lesions which may provide insight into the role of immune surveillance in the early phases of malignant initiation, transformation and progression of ovarian cancer.

MATERIAL AND METHODS

Human Tissues

Ten formalin-fixed paraffin-embedded (FFPE) tissue blocks of fallopian tube were retrieved from the surgical pathology files of the Department of Pathology, Spedali Civili of Brescia (Brescia, Italy). Patients underwent surgical removal of the fallopian tubes for non-cancer related conditions. Clinical details of the patient cohort are shown in Table 1. Haematoxylin & eosin (H&E) stained slides were reviewed by a pathologist (LA) in order to exclude the presence of any neoplastic or inflammatory conditions. Representative sections of the fallopian isthmus and fimbriae were available from each patient. In addition, three fragments of fresh fallopian tube were collected from two pre-menopausal and one post-menopausal patient who underwent total hysterectomy with bilateral salpingo-oophorectomy for surgical treatment of uterine leiomyoma. The study was approved by the local Ethics Committee.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on four microns FFPE tissue sections. The list of primary antibodies and detection systems is shown in Table 2. For single staining, diaminobenzidine (DAB, Dako, Glostrup, Denmark) was used as the chromogen and sections were counterstained with Meyer's Haematoxylin. For double immunohistochemistry, after completing the first immune reaction using DAB, the second immune reaction was visualized using Mach 4 MR-AP (Biocare Medical, Concord, CA, USA), followed by Ferangi Blue (Biocare Medical) as chromogen. Double

immunofluorescence sections were visualized with fluorescent labeled Texas Red- and FITC-conjugated isotype-specific secondary antibodies (Table 2). In order to compare the immune cell distribution in different parts of the fallopian tube, two pathologists (LA and WV) evaluated the intraepithelial [IE], lamina propria [LP] and muscular wall [MW], separately (Table 3). Immune cell density of dominant populations was initially determined using digital microscopy. The absolute cell count was quantified automatically using a custom-programmed script in Cognition Network Language based on the Definiens Cognition Network Technology platform (Definiens AG, Munich, Germany). In brief, immunostained slides of fallopian tube sections from the isthmus were digitalized using an Aperio ScanScope CS Slide Scanner (Aperio Technologies, Vista, CA, USA) at 40× magnifications and analyzed using Tissue Studio 2.0 (Definiens AG). The quantitative scoring algorithm was customized using commercially available templates and was created to match the requirements of the staining parameter in the fallopian tube including Manual ROI (region of interest) Selection (Draw Polygon). Immune cell counts are expressed as the number of positive cells/mm2 of fallopian tube area and as a ratio between positive cells and the total nucleated cells identified in the fallopian tube (Table 4). For immune cells showing intraepithelial localization, an additional cell count was performed at 40x magnification and expressed as a ratio of immune cells/epithelial cells, by counting at least 500 epithelial cells and excluding areas where there was epithelial overlay (Table 5).

Flow cytometry

Fresh fragments of human fallopian tubes were initially washed with saline solution to eliminate the large majority of circulating mononuclear cells. Then they were finely dissected, minced and digested using 1 mg/mL collagenase IV (Sigma–Aldrich, Dorset, UK) in PBS for 2 h at 37 °C. Digested tissue was filtered through 70 μm and 40 μm filters. Filtered material containing the fallopian tube single cell suspension was centrifuged twice for 10 min at 1500 rpm and stained with a mixture of the following monoclonal antibodies (mABs): iNKT-FITC, CD16-PE, CD8-PerCP-Cy5.5, CD3-PE-Cy7, CD45RA-APC-H7, CD4-V450 and CD45-V500 (all from Becton Dickinson, Franklin Lakes, NJ, USA) and CD56-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). At least 50000 lymphocytes were acquired from each sample using a FACSCanto II system (Becton Dickinson), while analyses were performed with FlowJo software version 8.8.7 (TreeStar, Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed using the GraphPrism program. For comparison of the number of the different types of intraepithelial immune cells according to menopausal status (pre- vs post-) and to tubal site (fimbriae vs isthmus), a Student's *t*-test was used. $P < 0.05$ was considered statistically significant.

RESULTS

Occurrence, density and distribution of immune cells in normal fallopian tube

Prior to this study we had observed that normal human fallopian tube regularly contains $CD45^+$ immune cells (Figure 1A). Based on a digital microscopy (DigMic) approach using Definiens Tissue Studio (Figure 2), we determined a density of $CD45^+$ immune cells

ranging from 147 to 743/mm2 (Table 4). In all samples we observed that CD45$^+$ cells were distributed in the epithelium, lamina propria and muscular wall (Figure 1B–D). In the epithelium the CD45$^+$ cells were intermingled with secretory and ciliated cells lying just above the basement membrane. In contrast, in the lamina propria and wall of the tube they were sparse or occasionally formed small aggregates that frequently surrounded blood vessels. Significantly, double immunohistochemistry for CD45 coupled with either estrogen or progesterone receptor showed no expression of hormones receptors by immune cells (Figure 1E–F). The morphology of CD45$^+$ cells was extremely variable, including round to oval cells admixed to cells with irregular morphology (i.e spindle or dendritic-like), thus indicating cell heterogeneity. To shed light on the identity and function of the immune cells in the epithelium, lamina propria and muscular wall of the fallopian tube, we undertook a comprehensive investigation using immunohistochemistry, immunofluorescence and flow cytometry.

CD8$^+$ intraepithelial T-cells represent the dominant lymphoid subset

Among B- and T-lymphoid cells, CD3$^+$ T-cells represented the dominant population, as validated by DigMic (Table 4 and Figure 3A). They were evenly distributed in all samples and localized in the epithelium as single cells intermingled between epithelial cells just above the basement membrane. In contrast CD20$^+$ B-cells were less frequently detected in the epithelium, lamina propria or wall (Tables 4–5 and Figure 3B). In the epithelium the average ratio of immune cells to epithelial cells was 1/16 for CD3$^+$T-cells and 1/64 for CD20$^+$B-cells respectively (Table 5) and the ratio between intraepithelial B-cells and T-cells was approximately 1/4. In the lamina propria and muscular wall, T- and B-cells were found as single scattered cells; in the muscular wall they also presented as perivascular lymphoid aggregates. Among T-cells, CD8$^+$ cells were the most abundant subset, especially in the epithelium, while CD4$^+$ cells were poorly represented (Tables 4–5 and Figure 3C–D). Accordingly, the average ratio of immune cells to epithelial cells was 1/400 for CD4$^+$ T-cells and 1/15 for CD8$^+$ T-cells respectively (Table 5), with a CD4$^+$/CD8$^+$ ratio of 1/25.

The prevalence of CD8$^+$ cells was confirmed by a flow cytometric analysis on single cell suspensions of fallopian tube (Figure 3M). The “global” CD4$^+$/CD8$^+$ ratio on flow cytometry was similar to the one found on the whole tubal sections analyzed with DigMic but significantly lower than the ratio observed in the epithelium on the tissue sections (1/4 to 1/5 versus 1/25), suggesting that CD8$^+$ T-cells preferentially home within the epithelium. Significantly, the vast majority of T-cells lacked CD45RA expression, suggesting a memory phenotype. According to double immunohistochemistry (Figure 3E) and immunofluorescence analysis (not shown) we identified in the epithelium a fraction of intraepithelial CD3$^+$ cells expressing the NK cell marker CD56. Flow cytometric characterization of CD3$^+$CD56$^+$ cells demonstrated that their percentage was highly variable from sample to sample (10 to 60% of CD3$^+$ cells), with most of the cells displaying an immunophenotype characteristic of effector memory cytotoxic T cells (CD8$^+$CD45RA$^-$). On the other hand, the presence of NKT cells in the CD3$^+$CD56$^+$ population was excluded by negativity for the NKT specific invariant Vα24-JαQ TCRα chain[15] (not shown).

By flow cytometry we also observed a subset of $CD3^{-}CD8^{+}$ cells (not shown). This finding was confirmed by double immunohistochemistry on FFPE sections (Figure 3F). These cells represent a fraction of $CD16^{hi}CD56^{dim}$ NK cells with low density surface expression of CD8[16].

On FFPE sections, the large majority of fallopian tube $CD3^{+}$ T-cell population was positive for T-bet (Figure 3G) and negative for nuclear Foxp3 (Figure 3H), indicating that virtually all of them were characterized by a type 1 polarization[17, 18] and that regulatory T-cells[19] are almost completely absent in the normal fallopian tube. Recently a new antibody has been shown to be capable of identifying $TCR\gamma\delta^{+}$ T-cells on FFPE[20]. We were able to confirm this data using this reagent to assess the presence of intraepithelial $\gamma\delta^{+}$ T-cells in the small bowel (unpublished data). In the fallopian tube $TCR\gamma\delta^{+}$ T-cells were rare and distributed as single intraepithelial T-cells (Figure 3I). Accordingly, the average ratio with epithelial cells was of 1/120, whereas the ratio of $TCR\gamma\delta^{+}/CD3^{+}$ T-cells was approximately of 1/8. This finding was confirmed by immunohistochemistry for TCRαβ that marked the large majority of intraepithelial lymphocytes (Figure 3J). Scattered $TCR\gamma\delta^{+}$ T-cells were also present in the lamina propria or in vessels of the wall of the tube. Based on the finding that most T-cells were represented by the $CD8^{+}$ subset, we extended the analysis by performing double immunohistochemistry for CD3 coupled with Granzyme-B or Perforin and could demonstrate that a significant proportion of intraepithelial T-cells contained these cytotoxic molecules (Figure 3K–L).

Occasional $CD138^{+}$ plasma cells were identified as scattered single cells in the lamina propria and tubal wall (not shown).

$CD11c^{+}$ dendritic cells and $CD163^{+}$ macrophages represent the predominant innate cell populations

On CD45 stained sections, we observed that a large fraction of positive cells showed irregular, non-lymphoid morphology, suggesting that they may be resident cells of the mononuclear phagocyte system. Based on the DigMic analysis, CD11c, CD68 and CD163 reactivity was frequently observed (Table 4). In tissue sections, this set of markers can identify human macrophages (CD11c, CD68 and CD163[Figure 4A–C]) and dendritic cells (CD11c and CD68). By double immunohistochemistry for CD163 and CD11c we could identify distinct cell populations that were differentially distributed in the epithelium, lamina propria and wall of the tube (Table 6). In the epithelium, the highest fraction was represented by $CD11c^{+}CD163^{-}$ cells (Figure 4D), while single positive $CD11c^{-}CD163^{+}$ cells and $CD11c^{+}CD163^{+}$ double positive cells were more rare. $CD11c^{+}CD163^{-}$ cells were regularly and evenly distributed between epithelial cells above the basal lamina and showed long intraepithelial cytoplasmic projections (Figure 4D, insert). In the lamina propria and wall, the most abundant population was represented by double positive $CD11c^{+}CD163^{+}$ cells, while single positive $CD11c^{-}CD163^{+}$ and $CD11c^{+}CD163^{-}$ cells were less frequently observed (Figure 4E). We extended the analysis of dendritic cells by staining for the Langerhans cells marker CD207(Langerin) and the plasmacytoid dendritic cells marker CD303(BDCA2). $CD207(Langerin)^{+}$ Langerhans cells were very rare but were regularly detected in the epithelium and lamina propria (Figure 4F), whereas $CD303(BDCA2)^{+}$

plasmacytoid dendritic cells were only occasionally found within the lumen of blood vessels in the lamina propria and tubal wall (Figure 4G).

Among innate immune cells, we also identified $CD56^{+}CD3^{-}$ NK cells in the epithelium, lamina propria and wall; in the epithelium they were evenly distributed between the epithelial cells with an average ratio of immune to epithelial cells of 1/70 (Table 5 and Figure 4H). Interestingly, flow cytometric analysis of fallopian tube fragments showed that at least half of NK cells had a negative/low expression of CD16, suggesting a derivation from the $CD56^{hi}$ compartment (data not shown).

$CD117^{+}$ mast cells and $CD66b^{+}Elastase^{+}$ neutrophils were identified respectively as scattered single cells and intravascular cellular groups in the lamina propria and tubal wall (not shown).

Site- and hormone-dependent variation of fallopian tube immune cells

Based on double immunohistochemistry for CD45 coupled with antibodies recognizing estrogen and progesterone receptors, we failed to detect hormone receptor expression on fallopian tube leucocytes, however, the epithelial cells strongly expressed these receptors (Figure 1E–F). Comparison of the intraepithelial number of distinct immune cells according to menopausal status (premenopausal vs postmenopausal status) and localization (fimbriae vs isthmus) is reported in Table 5. We found significant differences in the number of T-helper cells ($p<0.0027$), B-cells ($p<0.0003$) and macrophages ($p<0.034$, according to menopausal status, with T-helper cells and B-cells being more abundant in postmenopausal women while macrophages were more abundant in premenopausal women (data not shown). The only significant difference according to the location in the tube was found for intraepithelial NK cells ($p<0.0001$) which were more abundant in the isthmus (data not shown).

DISCUSSION

In this study we describe the identity and distribution of immune cells in the normal human fallopian tube by immunohistochemistry, immunofluorescence and flow cytometry, using a large panel of lineage and subset-specific markers. This is a preliminary analysis which lays the groundwork for subsequent studies correlating immune cell populations with the phases of the normal menstrual cycle, various infectious diseases and ovarian serous neoplasia given the emerging role of the fallopian tube as the site of ovarian carcinoma precursor lesions.

We found that the human fallopian tube contains a heterogeneous population of innate and adaptive immune cells including lymphocytes, macrophages, NK cells and dendritic cells. In terms of distribution, $CD45^{+}$ immune cells are found in the epithelium, lamina propria and muscular wall. By quantitative analysis using digital microscopy, we found that T-cells represent the most abundant lymphoid population. Intraepithelial T-cells are mainly $TCR\alpha\beta^{+}$ and express T-bet and lack Foxp3 in keeping with a Th1 type polarization[17–19]. As previously reported, we confirmed that intraepithelial T-cells are mostly $CD8^{+}$[21] cells and contain cytoplasmic perforin and granzyme B. Significantly, a minor fraction of

intraepithelial $CD3^+$ T-cells expressed TCRγδ. $TCR\gamma\delta^+$ T-cells are resident T-cells in skin, small and large intestine, lung and male and female reproductive organs[22, 23]. In these sites $TCR\gamma\delta^+$ T-cells play an important role in maintaining skin and mucosal homeostasis and are involved in regulation of inflammation, tissue repair, protection against infectious agents and elimination of transformed cells[24, 25]. In the female reproductive organs $TCR\gamma\delta^+$ cells have been isolated from the endometrium, including decidua, cervix and vagina[23] where they may exert a protective role against viruses[26]. The percentage of intraepithelial $TCR\gamma\delta^+$ T-cells in the fallopian tube is much lower in comparison to other types of mucosae, especially the small intestine, where they constitute up to 60% of intraepithelial lymphocytes[27]. Similarly, in the endometrium, $TCR\gamma\delta^+$ T-cells represent a small fraction of the intraepithelial T-cells[28]. By double immunohistochemistry and immunofluorescence we found that a fraction of intraepithelial T-cells co-expressed CD56. Although CD3/CD56 co-expression is a typical characteristic of NKT cells[29, 30] we excluded the presence of this cell subset by specific flow cytometric staining. In contrast, the vast majority of $CD3^+CD56^+$ cells were also $CD8^+CD45RA^-$, suggesting that most of the infiltrating lymphocytes are effector memory cytotoxic cells.

We also detected $CD3^-CD56^+$ NK cells, mainly in the epithelium. Using flow cytometry of single cell suspensions, it was possible to demonstrate that $CD56^{hi}CD16^-$ represents the dominant subset, as previously suggested[31], but that in the $CD56^{dim}CD16^{hi}$ fraction also contains a considerable number of $CD8^{lo}$ expressing cells. Among innate immune cells, the fallopian tube contains numerous $CD163^+$ macrophages and $CD11c^+$ dendritic cells. When analyzed by location using double immunohistochemistry, $CD11c^+CD163^-$ predominate in the epithelium, whereas $CD11c^+CD163^+$ cells were more abundant in the lamina propria and the wall of the tube. In contrast, $CD11c^-CD163^+$ macrophages were the least represented subset in the epithelium and non-epithelial compartments. CD11c was originally described as a dendritic cell marker[32], however, its specificity on sections of human tissues is questionable since anti-CD11c antibodies show reactivity to various macrophage populations. Significantly, high levels of CD11c can be found on mucosal macrophages[33].

In view of these findings we also tested other dendritic cell populations, namely plasmacytoid dendritic cells and Langerhans cells. It was previously reported that plasmacytoid dendritic cells are abundant in the fallopian tube[21]. We were unable to confirm this finding as we found $CD303(BDCA2)^+$ cells only occasionally in the lumen of blood vessels. Lack of plasmacytoid dendritic cells was also confirmed by the absence of $CD123^+$ and $CD2AP^+$ cells showing plasmacytoid morphology (not shown). Langerhans cells are the predominant antigen-presenting cells in epithelial tissues and selectively express CD207(Langerin) receptor[34]. The occurrence of Langerhans cells has previously been reported throughout the female reproductive organs, especially in the vagina[35], cervix[36] and endometrium[37]. Electron microscopy and immunohistochemistry for CD1a have identified Langerhans cells also in the fallopian tube[38]. By using the highly specific reagent CD207(Langerin), we could detect rare Langerhans cells, localized predominantly in the epithelium.

In summary, the findings in this study describing the heterogeneous population of immune cells in the fallopian tube lay the ground work for further investigation of the immune

system in ovarian serous carcinogenesis given the emerging role of the fallopian tube as the site of putative precursors of ovarian serous carcinoma. This is particularly provocative in view of the theory of incessant ovulation which implicates injury of the ovarian surface epithelium at the time of ovulation as contributing to malignant transformation by virtue of associated inflammation and repair. Conceivably macrophages can be an important component of this process by inducing DNA damage in tubal epithelial cells leading to the development of ovarian carcinoma [39] as borne out by a study reporting that the number of $CD68^+$ macrophages detected in the fallopian tube increases from benign conditions to malignant ones[40]. Moreover, a recent study indicates that $TCR\gamma\delta^+$ lymphocytes can efficiently kill human ovarian potential cancer stem-like cells through IL-17 production. This could represent a promising tool for immunotherapy in ovarian cancer[41]. Accordingly, further analysis of the identity and composition of immune cells in the fallopian tube and their relationship to ovarian cancer precursors is warranted as this may provide a potential approach to the development of immunotherapy aimed at early neoplastic lesions which are more likely to respond to immunotherapy than established cancers.

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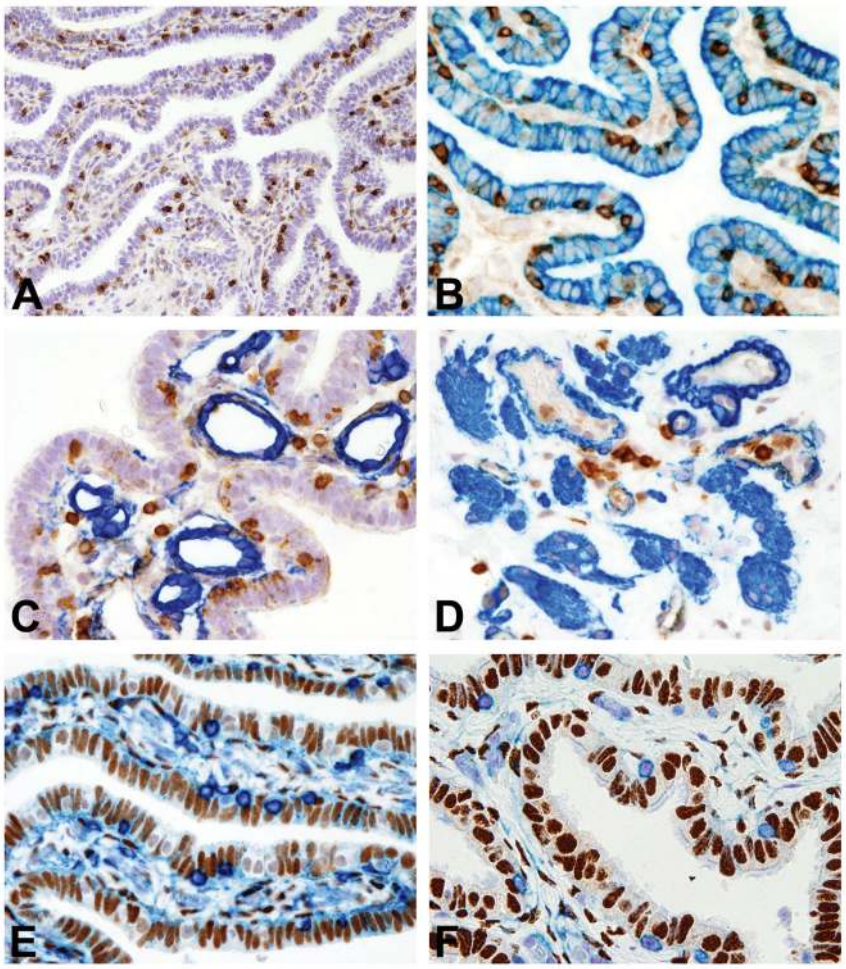

Figure 1. Localization and hormone receptor expression of resident leucocyte populations in normal fallopian tube

Sections are from FFPE normal fallopian tube (FT) (**A–F**). **A** illustrates $CD45^+$ immune cells in a FT isthmic section; **B** illustrates CD45(brown)/CK7(blue) double immunostaining showing $CD45^+$ immune cells intermingled with $CK7^+$ epithelial cells; **C** illustrates CD45(brown)/CD34(blue) double immunostaining showing $CD45^+$ immune cells in close proximity to $CD34^+$ vessels of the FT lamina propria; **D** illustrates CD45(brown)/smooth muscle actin (blue) double immunostaining showing $CD45^+$ immune cells in close proximity to smooth muscle $actin^+$ smooth muscle fibers/vessels of the FT muscular wall; **E–F** illustrate double immunostaining with CD45 (E–F, blue) and ER (brown, E) and PgR (brown, F) showing no expression of hormones receptors by immune cells. Sections are counterstained with Meyer's haematoxylin. Magnification: 20× (A) and 40× (B–F).

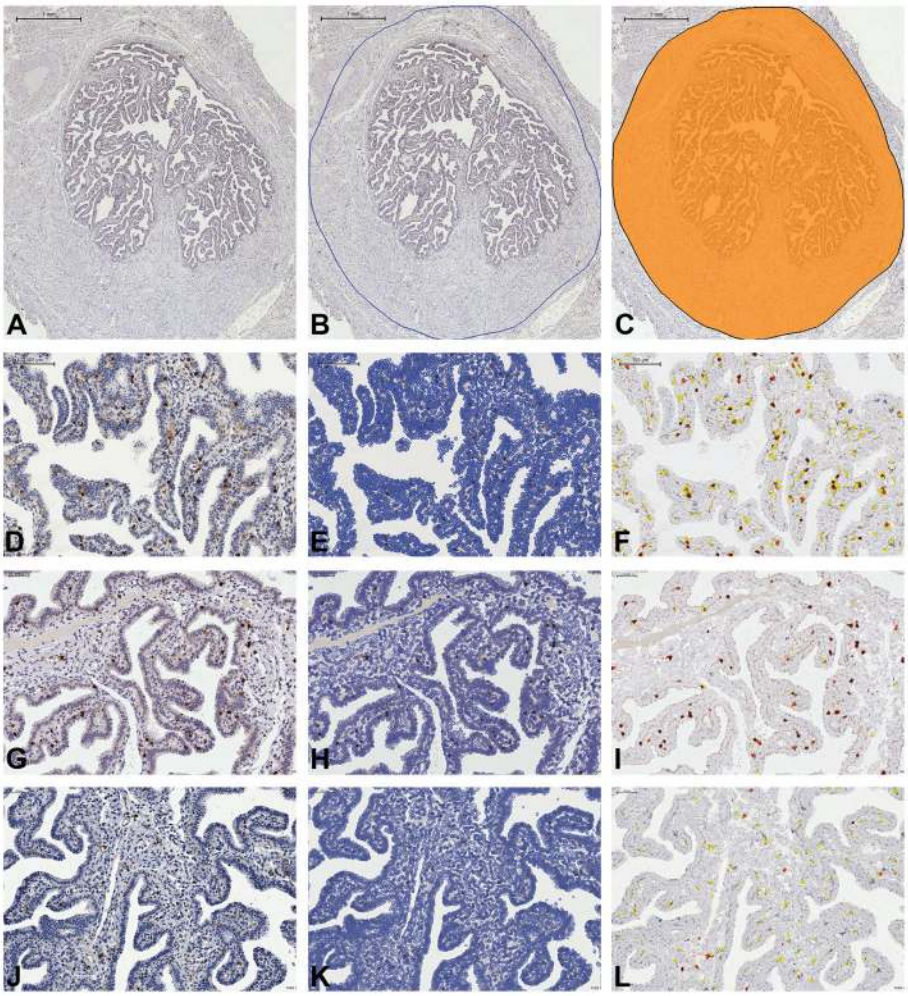

Figure 2. Digital Microscopy analysis of the main immune cell populations in the normal fallopian tube (FT)

Sections are from FFPE normal fallopian tube and are digitalized by Aperio Scanscope and processed by Tissue Studio Definiens. **A–C** illustrate ROI (region of interest) selection: **A** illustrates the digital image of whole isthmic section of FT; **B** illustrates the manual selection of the ROI; **C** indicates the ROI (orange color) submitted to Tissue Studio Definiens for image analysis (Bar scale: A–C 1mm). **D–F**, **G–I**, **J–L** are matched native (D, G and J) and processed for nuclear identification (E, H and K) and signal detection (F, I and L; white cells are negative cells whereas yellow, orange and red cells correspond to positive cells with different signal intensity) images (Bar scale: D–F 100μm; G–L: 50 μm).

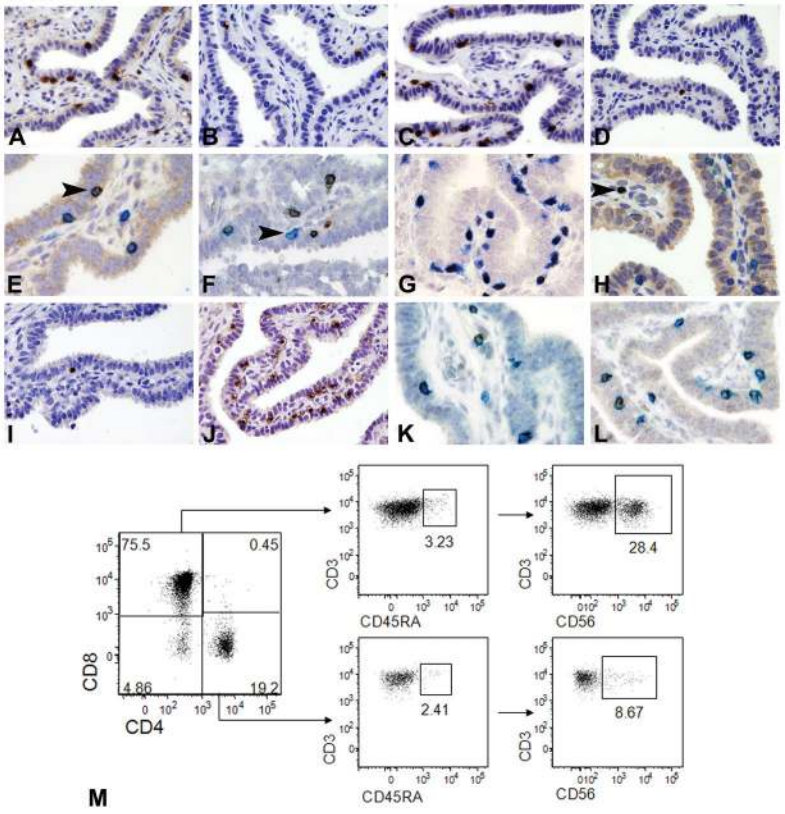

Figure 3. Cellular populations of the adaptive immune system in the normal fallopian tube

Sections from FFPE normal fallopian tube are analyzed by immunohistochemistry (**A–L**) whereas single cell suspension of fresh normal fallopian tube is analyzed by flow cytometry (M). **A–D** illustrate the main lymphoid populations detected in the intraepithelial compartment (IE) including $CD3^+$ T-cells (A), $CD20^+$ B-cells (B), $CD8^+$ cytotoxic T-cells (C) and $CD4^+$ T-helper cells (D). **E** illustrates CD3(blue)/CD56(brown) double immunostaining showing co-expression of CD56 NK marker by a $CD3^+$ T-cells (arrow head). **F** illustrates CD3(brown)/CD56(blue) double immunostaining showing the presence of $CD3^+CD8^+$ T-cells (prevalent subtype) and $CD3^-CD8^+$ cells (arrow head), likely representative of $CD8^+$ NK subset. **G** illustrates CD3(blue)/T-bet (brown) double immunostaining showing the prevalence of $Tbet^+CD3^+$ T-cells. **H** illustrates CD3(blue)/Foxp3(brown, nuclear) double immunostaining showing absence of $Foxp3^+$ regulatory T-cell in the IE compartment; arrow head indicates a single $CD3^+Foxp3^+$ regulatory T-cells in the lamina propria. **I–J** show comparison between IE TCR$\gamma\delta^+$ (I) and TCR$\alpha\beta^+$ T-cells (J). **K–L** illustrate double immunostaining with CD3 (K–L, blue) and Granzyme B (K, brown) and Perforin (L, brown) showing the presence of those cytotoxic molecules in $CD3^+$ T-cells. Sections are counterstained with Meyer's haematoxylin. Magnification: 40× (A–D, I–J) and 60× (E–H, K–L). **M** shows flow cytometry characterization of T lymphocytes on digested tubal tissue. Left plot: CD4/CD8 ratio; central and right plots: percentage of $CD45RA^+$ and $CD56^+$ cells, respectively, in the $CD8^+CD4^-$ and $CD4^+CD8^-$ gates (The panel is representative of three different samples).

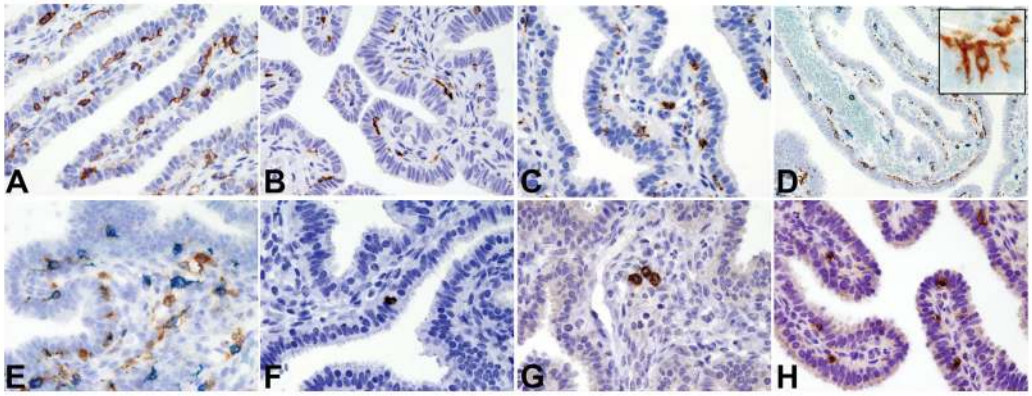

Figure 4. Cellular populations of the innate immune system in the normal fallopian tube

Sections are from FFPE normal fallopian tube (A–H) and stained for CD11c (A), CD68 (B) and CD163 (C). **D–E** illustrate CD163(blue)/CD11c(brown) double immunostaining showing the presence respectively of $CD11c^{+}CD163^{-}$ cells in the intraepithelial compartment (D; insert showing intraepithelial cytoplasmic projections of dendritic cells) and of $CD11c^{+}CD163^{+}$ cells admixed to $CD11c^{-}CD163^{+}$ cells in the lamina propria (E); **F** illustrates CD207/$Langerin^{+}$ intraepithelial Langerhans cell; **G** illustrates occasional CD303/$BDCA2^{+}$ plasmacytoid dendritic cells in vessels of the lamina propria; **H** illustrates intraepithelial $CD56^{+}$ NK cells. Sections are counterstained with Meyer's haematoxylin. Magnification: 40× (A–C and E–H) and 20× (D).

Table 1

Clinical details of the patients cohort

Patient ID	Age (yrs)	Menopausal status	Surgical Procedure/Indication
#1	63	Post	TAH-BSO/Leiomyomas
#2	67	Post	TAH-BSO/Leiomyomas
#3	67	Post	TVH-BSO/Prolapse
#4	73	Post	TVH-BSO/Prolapse
#5	63	Post	TVH-BSO/Leiomyomas
#6	32	Pre	SA/Sterilization
#7	35	Pre	SA/Sterilization
#8	30	Pre	SA/Sterilization
#9	26	Pre	SA/Sterilization
#10	35	Pre	SA/Sterilization

Post: post- menopausal; TAH-BSO: total abdominal hysterectomy with bilateral salpingo-oophorectomy; TVH-BSO: total vaginal hysterectomy with bilateral salpingo-oophorectomy; Pre: pre-menopausal; SA: salpingectomy

Table 2

Details of primary antibodies and detection systems

Primary antibody	Source	Clone	Dilution	Company	Detection system
CD2AP	Ms IgG1	B-4	1:1000	SC	E
CD3	Rb mAb	SP7	1:100	TS	E
CD3 (IF)	Rb mAb	SP7	1:100	TS	Rb FITC
CD4	Ms IgG1	4B12	1:50	NL	NP
CD8	Ms IgG1	C8/144B	1:30	DK	E
CD11c	Ms IgG2	5D11	1:50	Mo	E
CD20	Ms IgG2a	L26	1:250	DK	E
CD45	Ms IgG1	X16/99	1:500	NL	NP
CD56	Ms IgG1	123C3.D5	1:30	TS	E
CD56 (IF)	Ms IgG1	123C3.D5	1:30	TS	Ms biot/Sav-TXR
CD66b	Ms IgM	G10F5	1:200	Bi	NP
CD68	Ms IgG3	PG-M1	1:200	DK	E
CD117	Rb pAb	polyclonal	1:50	DK	E
CD123	Ms IgG2a	7G3	1:50	BD	NP
CD138	Ms IgG1	MI15	1:100	DK	E
CD163	Ms IgG1	10D6	1:50	TS	E
CD207 (Langerin)	Ms IgG2b	12D6	1:150	VL	E
CD303 (BDCA2)	Ms IgG1	124B3.13	1:75	De	NP
Calretinin	Rb pAb	polyclonal	1:200	I	E
Neutrophil Elastase	Ms IgG1	NP57	1:150	DK	NP
Estrogen Receptor	Rb mAb	SP1	1:100	TS	E
Foxp3 biot	Rat IgG2a	FJK-16s Set	1:200	eB	RP
Granzyme B	Ms IgG2a	11F1	1:100	NL	NP
Perforin	Ms IgG1	5B10	1:20	NL	E
Progesterone Receptor	Ms IgG1	PgR	1:200	DK	E
T-bet	Ms IgG1	4B10	1:50	SC	NP
TCRβF1	Ms IgG1	8A3	1:30	TS	NP

Primary antibody	Source	Clone	Dilution	Company	Detection system
TCRCγM1	Ms IgG1	γ3.20	1:400	TS	NP

IF: immunofluorescence; Rb mAb/pAb: rabbit monoclonal or polyclonal antibody; Ms: mouse antibody; biot: biotinylated antibody.

Detection system: E: EnVision (Dako); Rb FITC: Swine anti-rabbit FITC (Dako); NP: Novolink polymer (Novocastra Laboratories); Ms biot: goat anti-mouse biotynilated IgG1, Sav-TXR: Streptavidin TXR-conjugated (Southern Biotecnology*); RP: Rat-on-mouse HRP-polymer (Biocare Medical).

Company addresses: *SouthernBiotech, Birmingham, AL, USA; SC: Santa Cruz Biotechnology, Santa Cruz, CA, USA; TS: Thermo Scientific, Fremont, CA, USA; NL: Novocastra Laboratories, Newcastle upon Tyne, UK; DK: Dako, Glostrup, Denmark; Mo: Monosan, Uden, The Netherlands; Bi: BioLegend, San Diego, CA, USA; BD: BD Biosciences, San Jose, CA, USA; VL: Vector Laboratories, Burlingame, CA, USA; De: Dendritics, Lyon, France; I: Invitrogen, Carlsbad, CA, USA; eB: eBioscience, San Diego, CA, USA.

Table 3

Immunohistochemical profile and localization of the main immune cell types of the fallopian tube

ADAPTIVE		
Immune cell type	**Immunophenotype**	**Tissue localization**
T-cells	$CD3^{+}CD20^{-}$	IE, LP, MW
Cytotoxic T-cells	$CD8^{+}CD3^{+}$	IE, LP, MW
T-helper cells	$CD3^{+}CD4^{+}$	IE, LP, MW
Regulatory T-cells	$CD3^{+}Foxp3^{+}$	LP, MW
TCR$\gamma\delta^{+}$ T-cells	TCRCγM1^{+}	IE, LP, MW
B-cells	$CD3^{-}CD20^{+}$	IE, LP, MW
Plasma cells	$CD138^{+}$	LP, MW
INNATE		
Macrophages	$CD68^{+}CD163^{+}$	IE, LP, MW
NK cells	$CD3^{-}CD56^{+}$	IE, LP, MW
Plasmacytoid DCs	CD303(BDCA2)$^{+}$	LP*, MW*
Langerhans cells	CD207(Langerin)$^{+}$	IE, LP
Other type of DCs	$CD11c^{+/-}CD163^{+/-}$	IE, LP, MW
Mast cells	$CD117^{+}$	LP, MW
Neutrophils	$CD66b^{+}$Neutr El^{+}	LP*, MW*

DCs: dendritic cells; IE: intraepithelial; LP: lamina propria; MW: muscular wall; *: almost exclusively intravascular localization; Neutr El: neutrophil elastase

Table 4

Digital Microscopy cell analysis of immune cells of the fallopian tube

IHC Marker	Positive cells/mm^2 (range)	Positive cells/total nucleated cells (range)
CD45	433 (147–743)	1/18 (1/49–1/11)
CD3	120 (110–136)	1/69 (1/83–1/56)
CD4	35 (15–75)	1/175 (1/555–1/82)
CD8	160 (94–196)	1/47 (1/76–1/36)
CD11c	278 (200–364)	1/21 (1/29–1/17)
CD20	27 (3–58)	1/270 (1/3333–1/135)
CD68	254 (178–320)	1/31 (1/45–1/24)
CD163	306 (204–368)	1/30 (1/50–1/25)
TCR CγM1	11 (10–21)	1/555 (1/833–1/370)

IHC: immunohistochemistry

Table 5

Ratio between different types of immune cells and epithelial cells in the IE compartment

Immune cell type	OA Ratio	Pre- vs Post-menopause *(p-value)*	Fimbriae vs Isthmus *(p-value)*
T-cells	1/16	*0.53*	*0.41*
Cytotoxic T-cells	1/15	*0.79*	*0.07*
T-helper cells	1/400	*0.0027**	*0.94*
B-cells	1/64	*0.0003**	*0.14*
TCRγδ+ T-cells	1/120	*0.93*	*0.46*
NK cells	1/70	*0.67*	*0.0001**
Macrophages	1/12,5	*0.034**	*0.08*
Langerhans cells	1/625	*0.62*	*0.88*

IE: intraepithelial; OA: overall average; Pre-; premenopausal; vs: versus; post-: post-menopausal; F: fimbriae; I:isthmus:

* statistically significant

Table 6

Immunophenotype and distribution of large stellate/spindle cells in the fallopian tube

Subsets	IE	non-IE
$CD11c^{+}CD163^{+}$	+	++
$CD11c^{+}CD163^{-}$	++	+
$CD11c^{-}CD163^{+}$	+	+

IE: intraepithelial; +: minor subset; ++: major subset