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Transfusion Associated Microchimerism: The Hybrid Within

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Abstract

Microchimerism, the coexistence of genetically disparate populations of cells in a receptive host, is well described in both clinical and physiological settings, including transplantation and pregnancy. Microchimerism can also occur following allogeneic blood transfusion in traumatically injured patients, where donor cells have been observed decades after transfusion. To date, transfusion-associated microchimerism (TA-MC) appears confined to this clinical subset, most likely due to the immune perturbations that occur following severe trauma that allow foreign donor cells to survive. TA-MC appears to be unaffected by leukoreduction and has been documented following transfusion with an array of blood products. The only significant predictor of TA-MC to date is the age of red cells, with fresher units associated with higher risk. Thus far, no adverse clinical effect has been observed in limited studies of TA-MC. There are, however, hypothesized links to transfusion-associated graft vs. host disease (TA-GvHD) that may be unrecognized and consequently under-reported. Microchimerism in other settings has gained increasing attention due to a plausible link to autoimmune diseases, as well as its diagnostic and therapeutic potential vis-a-vis ante-natal testing and adoptive immunotherapy, respectively. Furthermore, microchimerism provides a tool to further our understanding of immune tolerance and regulation.

Keywords

Microchimerism; transfusion; chimerism; trauma; immunity; immune tolerance

INTRODUCTION

The word chimera is named for the mythological beast, originally referenced in Homer's Iliad. This fire-breathing creature comprised the body of a lioness, the head of a goat and the tail of a transformed serpent. A sibling of both the Lernaean Hydra and Cerebrus, the multi-headed hound, a sighting of the chimera was said to be ominous of disaster.

The chimera in Science builds on the notion of a hybrid animal to represent an organism of mixed genetic origin. Specifically, chimerism refers to the enduring co-existence of genetically disparate populations of cells within a single host. Unlike mosaicism, where the

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discordant cell lines originate from the same zygote, the chimeric cell is truly foreign or allogeneic, having originated outside the host.

Although the term microchimerism (MC) is frequently used interchangeably with chimerism, it is generally accepted that the term MC should be reserved for circumstances in which the foreign populations account for less than 5% of host cellular content, or more specifically of nucleated cells in the relevant organ or blood. These allogeneic or non-self cells elude the host immune system and persist at low levels. The clinical significance of enduring chimerism includes both adverse effects (e.g., autoimmune diseases[1–3]) as well as potential therapeutic benefits (e.g., tolerance induction, tissue regeneration and repair[4, 5]).

Two key components define MC: (1) “quantity”, indicating that the population is of sufficient magnitude to be detectable using laboratory screening and (2) “longevity” indicating that the cells are durable over time. The latter is somewhat arbitrary, contingent on a persistence that is deemed excessive for the initiating event, e.g. persistence of allogeneic cells beyond 3–6 months following pregnancy or blood transfusion is indicative of long-term MC. Short-term MC (likely representing delayed clearance of allogeneic cells) is significantly more frequent than long-term MC and does not necessarily predict long-term durability. Several studies have reported survival of donor leukocytes after transfusion, yet limited follow-up precliudes a clear definition of durable MC. [6, 7] Should levels of chimerism start off below the limits of detection and increase over time, eventually long-term MC may occur in the absence of short-term MC; this may reflect expansion of a minute population, which is only detectable months to years following allogeneic exposure.

CLINICAL SETTINGS

MC may be iatrogenic following solid organ-, bone marrow transplantation or blood transfusion; it may also arise physiologically through pregnancy, twinning or intergenerational transfer (see below). [1–5, 8–12] Sexual transmission and needle sharing have also been suggested, as possible mechanisms for MC, yet have not been well documented.

Fetal-Maternal Microchimerism

Pregnancy is the most common setting in which MC is encountered, given bidirectional transfer of cells and cell-free DNA between mother and fetus. This may be necessary to establish immune tolerance between the mother and fetus[11], as evidenced by the detectable presence of both fetal cells and cell-free DNA in the maternal circulation in the majority of pregnancies. Chimeric cells increase from first appearance at 4–5 weeks up until parturition, when rapid and near-complete cellular clearance occurs[13]. [8], Long-lived MC is observed in some parous women, and has in some circumstances been documented decades following delivery.

Terminology surrounding pregnancy-associated chimerism may be confusing: fetal-maternal microchimerism (FM-MC) is a generic term to denote MC that occurs during or subsequent to pregnancy, irrespective of directionality. In contrast fetal-MC (F-MC) refers to the persistence of fetal cells in the mother while maternal-MC (M-MC) refers to the persistence of maternal cells in the fetus.[11, 14] F-MC can occur between members of a multiple pregnancy: indeed, the original research in MC was spurred by observations of pathology in freemartins (female twin cattle) arising through in-utero hormone exposure from their male siblings. Furthermore, the seminal work performed by geneticist Ray Owens documented blood group concordance among fraternal twin cattle.[15] Similarly in humans, up to 8% of twins and 21% of triplets have been shown to exhibit blood group chimerism.[16]

F-MC has been an unanticipated finding in a number of studies[17–19], including in control groups that have been selected using women that self-report as never having been pregnant. This underscores that pregnancy need not be recognized for MC to take place; up to 30% of pregnancies end in fetal loss with 14% going unnoticed.[20] Both “occult” pregnancy as well as “vanished” or resorbed twins are more common than is appreciated and may result in durable MC. In one report, F-MC was attributed to a vanished twin 40 years after the implicated pregnancy.[21] The transfer of cells from progeny to mother also raises the possibility that fetal cells, which have persisted in the mother following an antecedent pregnancy, can be exchanged with a sibling during a subsequent pregnancy (intergenerational transfer[9]).

Durable FM-MC is not unique to term gestation and has been reported following both spontaneous and elective abortions. Increased rates of MC are observed in the latter, which may be attributable to fetal-maternal hemorrhage with increased volume of cellular transmission.[22] Other factors that appear to impact FM-MC include fetal and placental abnormalities, fetal aneuploidy and the length of time lapsed between delivery and investigation.[22] In contrast, parity does not appear to have a significant impact on rates of FM-MC.[23]

Transplantation- and Transfusion-associated Microchimerism

Transplantation-associated MC can be coincidental or imposed for therapeutic benefit. In solid organ transplantation, there is opportunity for systemic migration of foreign cells beyond the graft, despite localization of the donor organ (e.g., kidney, liver (reviewed in [12]). In hematopoietic stem cell (HSC) transplantation, systemic chimerism is an intended outcome with a goal being either complete or partial replacement of the recipient’s HSC compartment.

Blood transfusion is the most frequently performed allogeneic transplantation. Interest in the kinetics of transfused donor cells dates back to the 1970s when Schechter *et al.* demonstrated karyotypic evidence of donor leukocyte proliferation within 7 days of blood transfusion.[24] Subsequent studies, however, were unable to detect evidence of donor leukocyte survival beyond 6 days in immunocompetent recipients.[25] Many of the earlier studies were, however, constrained by limited sensitivity of the technologies at time of evaluation. It was approximately twenty years later when an attempt to extend Schechter’s work lead to a serendipitous finding that reshaped our understanding of TA-MC. Lee *et al.* [26] conducted a case control study that evaluated subjects transfused during elective surgery (cases) and compared these with a control group comprising patients that had been transfused for traumatic injury. The kinetics observed in the case group replicated that of the earlier experience: rapid clearance of 99.9% donor leukocytes was followed by a transient expansion of donor cells observed between days 2 and 7 with complete clearance by 7 to 14 days after transfusion.[26] In contrast, the majority (7 out of 10) of the “control” group unexpectedly developed durable TA-MC beyond 7 days. Follow-up studies have supported the finding that transfusion in patients following severe traumatic injury results in high rates (20–40% [27] of patients affected) of enduring (up to 60 years) TA-MC. This pertains to both combat casualty and civilian trauma settings.[28, 29]

With rare exceptions, durable TA-MC has remained peculiar to patients transfused for severe traumatic injury. [29–31] The few studies that have sought to evaluate other populations at “risk” for TA-MC have yielded negative results. Some of these studies have investigated populations that share features with the trauma setting, thereby offering plausible risk of TA-MC. Examples include studies of TA-MC in the HIV-infected population, as a paradigm of an immunosuppressive state[32], elective surgery as a model of iatrogenic “trauma”[26], and sickle cell anemia and thalassemia[33] reflecting chronic

transfusion risk. TA-MC was not significantly increased or durable in these patient populations.

A large cohort of adult and pediatric transfusion recipients were evaluated as part of the Transfusion Related Infections Study (TRIPS); 40 of the 207 (19%) adult and 44 of 202 (22%) of the pediatric transfusion recipients of leukoreduced and predominantly irradiated red cells and platelets, demonstrated transient TA-MC at 4 and/or 8-weeks post transfusion. However, on repeat testing only 12 (3%) showed TA-MC during the 2 months post-transfusion, none of which displayed durable chimerism.[34]

More recently, a study in South Africa, investigated whether TA-MC would occur in women transfused for peripartum hemorrhage. TA-MC was hypothesized to occur based on the overlap between the peripartum period and the trauma setting, given the acute blood loss, pain and similar immune perturbations that accompany parturition. However, despite a small sample size (n=22), the findings from this pilot study suggest that durable TA-MC does not occur in this population.[35]

Proposed reasons that place the trauma patient at greatest risk of durable TA-MC include the large number of unit exposures, which often include cellular components with relatively short periods of storage, as well as an altered immune response that occurs transiently following trauma.[36] This acute immunological imbalance is thought to predispose to a transient tolerance to allogeneic cells enabling low-level engraftment, thus manifesting as TA-MC. Both diminished innate and adaptive immune responses with transient T cell suppression and altered cytokine profiles are observed following injury. The latter is increasingly recognized as a complex, dynamic state in response to tissue damage, heightened stress, and the compounding contribution of exposure to microbial products from body site contamination. In addition predisposing genetic factors may account for variability of risk among transfusion recipients.[36]

The chimeric populations have been shown to comprise multi-lineage hematopoietic cells[37], supporting a hypothesis of stem cell engraftment; however, this has not been proven definitively.[26] Lee *et al.* evaluated chimeric cell phenotypes specifically in TA-MC using antibody-coated magnetic beads against $CD4^+$ and $CD8^+$ (T cells), $CD15^+$ (myeloid cells), and $CD19^+$ (B cells), and detected all 4 of these cell populations. Thus TA-MC is distinct from TA-GvHD, which involves excusive expansion of alloreactive donor lymphocytes targeting host histocompatibility antigens.

PREDICTORS OF TA-MC

Multiple blood product and patient characteristics have been examined in an attempt to predict TA-MC. In a cohort of 45 transfused trauma patients, 53% of which had short-term TA-MC,[10] Utter *et al.* examined multiple factors including recipient age, sex, type of injury, injury severity, volume of transfusion, presence of hypotension, pre-injury comorbidities (e.g. cardiorespiratory disease, diabetes, substance abuse, or malignancy), and time interval between injury and transfusion, none of which were shown to have a significant association with TA-MC. TA-MC also does not appear to be influenced by the age of the recipient at time of transfusion; in one case TA-MC occurred following transfusion of a fetus, (donor cells were detected 25 years following intrauterine transfusion).[38] TA-MC is also relatively common in young men transfused following combat injury.

TA-MC occurs independent of leukoreduction[29, 39] with similar rates of TA-MC having been demonstrated following transfusion with both leukoreduced and non-leukoreduced products and hence is not WBC dose dependent. In a substudy of a double-blinded

randomized-controlled trial evaluating the effect of leukoreduction on development of infection within 28 days of traumatic injury,[40] 9/32 (28.1%) patients in the non-leukoreduced arm had durable TA-MC while 13 of 35 (37.1%) in the leukoreduced arm developed TA-MC (p=0.43) on later follow-up.[31] This is not entirely unexpected; leukoreduction reduces white blood cells (WBCs) below 5×10^6 WBCs/component, but does not eliminate WBCs completely.

While donor recipient HLA mismatch has not been predictive of TA-MC in trauma patients in limited studies to date,[26] one retrospective study showed that transfusion of blood with at least one HLA haplotype shared between donor and recipient was more likely to result in TA-MC at 5–8 weeks of follow-up than receipt of mismatched blood (3/12 vs. 1/5, p=0.04).[6] However, the limited follow-up and transfusion shortly prior to renal transplantation limit the conclusion with respect to durable TA-MC.

Currently, many are advocating against use of older blood, citing concerns of heightened risk and reduced efficacy with prolonged storage. Reed *et al.* reported a significantly (p=0.024) different red cell storage time between non-TA-MC recipients (21±8.3 days) vs. TA-MC recipients (16.1±6.2 days).[28]Similarly the minimum storage time of packed red blood cells (pRBC) units was a median of 13 days in non-TA-MC recipients and 5 days [p=0.004] in TA-MC recipients.[28] Although this suggests that rates of TA-MC are inversely related to storage age, TA-MC has also been reported with older units (22 days following collection).[39]

The Immunology of Trauma, Transfusion and Microchimerism

Traumatic injury and transfusion are significant immunological stressors, triggering responses to the primary events themselves, as well as inducing major changes in the body's response to associated immunological challenges posed by secondary pathogen exposure and surgical intervention. The immune modulation resulting from these events forges a unique environment that appears critical for the development of TA-MC.

The immune perturbations that accompany trauma may explain why patients that have been transfused following injury are uniquely susceptible to TA-MC. This was illustrated in a case-control study of 63 transfused trauma patients where the patients' lymphocyte responses were compared with those of 10 non-transfused trauma patients and 10 healthy controls.[29] Lymphocyte responses were reduced in patients that had sustained traumatic injury, with even greater reduction in recipients who developed TA-MC. This trauma-induced immune hyporesponsiveness was not resolved at time of discharge. Furthermore, in another study, mixed lymphocyte reactions demonstrated a bidirectional hyporeactivity between donors and recipients that preceded transfusion.[41] Of note, recipients were shown to be least responsive to WBCs from the individual donors with whom they became chimeric. This strongly suggests a relative histocompatibility advantage of a particular donor in a particular recipient. In addition, TA-MC results from a single donor, despite potential exposure to multiple donors; this may be analogous to single donor stem cell engraftment observed in double cord blood transplantation.[29]

The immune response to trauma has been characterized through both evaluation of peripheral responses in humans as well as simulation of trauma using animal models. Following trauma, there are consistent increases in interleukin (IL)-6, IL-10, IL-1Ra, and IL-8; there may also be increases in tumor necrosis factor α and IL-4 yet the data in this regard are conflicted.[42–55] Other observed changes include increased blood levels of IgE and decreased IgM,[55–57] altered T cell effector function with increased regulatory T cell activity, decreased expression of HLA-DR and concomitant increases in IL-10 and IL-6 expression in monocytes.[58–65] A recent study demonstrated a genomic storm precipitated

by trauma with massive changes in RNA expression profiles, suggesting a major shift in immune responsiveness.[66] Murine models, designed to investigate the response to traumatic stresses such as burns, fractures, hemorrhage, and/or laparotomy (surgical stress), both support the human study findings demonstrating elevated IL-6 and IL-10, as well as show altered *ex vivo* functions of T cells and dendritic cells, with increased regulatory T cell activity.[67–75]

There are multiple factors that can influence the host immune response to injury and thereby contribute to donor cell survival. These include –but are not limited to- the extent of tissue damage, exposure to microbial components, hemorrhage, stress/neuroendocrine activation, the patient's age and sex.

Tissue damage can induce innate immune activation through several mechanisms. Alarmins, such as high-mobility group box 1 protein and heat-shock proteins, and damage-associated molecular patterns (DAMPS) such as mitochondrial DNA that are normally shielded in intracellular compartments can be released from necrotic cells and induce inflammation after injury.[76–78] Similarly, damage to the epithelium can allow for microbial entry, with recognition via innate immune receptors such as TLR4, while increased endothelial permeability can allow translocation of bacteria from the gastrointestinal tract into the blood stream.[79, 80]

Large volume hemorrhage may precipitate ischemia and later reperfusion injury compounding tissue damage and innate immune activation. In murine hemorrhage models where bleeding is induced either by depletion to a fixed volume or to a targeted blood pressure, short-term deficits in IL-2 production and T cell proliferative capacity have been observed.[81–83] Trauma also results in major endocrine and neurological changes that impact immunity. For example, the release of catecholamines has been shown to have an immunosuppressive effect leading to increased IL-10 production *in vivo* and *ex vivo*[84, 85] and inhibited responsiveness to lipopolysaccharide *ex vivo.*[86–88] Corticosteroids, released as part of the stress response, also lead to immunosuppression.[89–93]

The sex and age of the patient have a significant effect on the immune response to trauma: increased rates of death, sepsis, and multiple organ dysfunction syndrome have been demonstrated in men, with this sex difference modulated by patient age.[94–98] This difference appears to be regulated by sex hormones as manipulation in mice by oopherectomy, castration, or administration of androgens or estrogens, alters the response to trauma and septic challenges.[99, 100] Although significant differences in risk of TA-MC have not been reported between men and women or among different age groups, these factors may contribute in part to establishing an immunological environment that is favorable or unfavorable to development of TA-MC.

Allogeneic blood transfusion, itself, is a major immunological challenge (particularly when non-leukoreduced blood products are used). The immunosuppressive effect of allogeneic transfusion has long been recognized, and has been shown to confer both beneficial clinical effect, e.g. reduced graft failure after transplantation, [101–103] as well as adverse outcomes, e.g. increased cancer recurrence and susceptibility to infectious disease.[102, 104–109] This immunosuppression may also help to drive the development of microchimerism. In the non-trauma setting, transfusion of allogeneic blood can result in a proinflammatory allo-response, prompting both antibody and cellular responses against donor-specific antigens.[110–114] Ensuing alloimmunization may complicate future transfusions or solid organ transplantation.[112, 115, 116]

Historically, the initial response to severe injury was described as proinflammatory; referred to as the systemic inflammatory response syndrome (SIRS), this was thought to be

beneficial both to contend with exposure to pathogens that accompany injury, as well as to promote wound healing. A compensatory anti-inflammatory response syndrome (CARS), which follows this initial phase, was deemed important to reduce tissue damage associated with the inflammatory phase. However both excessive immune suppression and activation introduce risk of exacerbating tissue damage and contribute to development of multiple organ failure (MOF). [117–119]

In contrast to the SIRS/CARS model, two recent studies demonstrate that the development of an immunosuppressive cytokine milieu after trauma occurs rapidly, and may already be established by the time the patient arrives in the hospital. This immunosuppression coincides with the timing of blood transfusion, suggesting that the host environment at the time of transfusion may determine the survival of donor cells. In one study, 56 trauma patients were evaluated prospectively from arrival in the emergency room up to one year after injury, using serial blood samples.[120] The levels of 41 different immunomodulatory proteins were tracked and analyzed with clinical data using a multivariable approach. This demonstrated a complex and evolving response to trauma, which begins with a mixed, but predominantly anti-inflammatory response (figure 2). The balance between pro- and anti-apoptotic factors was also shown to be altered by trauma, with an initial pro-apoptotic balance shifting towards being anti-apoptotic 1–4 weeks after injury (figure 2). A number of factors involved in tissue remodeling, lymphocyte homeostasis, and endothelial activation, also become upregulated in the blood during this same timeframe (1–4 weeks) as the system restores itself (figure 2). An early mixed response is consistent with another recent study examining RNA expression profiles post trauma. [66]

TECHNIQUES FOR DETECTION OF MC

Detection of MC has inherent challenges: the target cellular population is minute and can represent as little as 1 chimeric cell per 10^6 WBC, which may be overwhelmed by the host's cellular burden. Therefore, detection of chimerism is rarely based on cell recognition such as in microscopy or by fluorescent in-situ hybridization (FISH),[121, 122] but on molecular signals from whole blood or enriched subpopulations of cells. Therefore, primers or target probes are needed that adhere uniquely to the chimeric minor population (See Table 1).

Early techniques largely employed gender discordance, through targeting the Y-chromosome, for evaluation of MC. This has inherent problems both for FM-MC as well as TA-MC. In FM-MC, a Y-chromosome probe restricts evaluation to a single male chimeric population in the maternal host, excluding female progeny. It also introduces both unexpected findings following occult pregnancy or abortion, as well as imprecision with respect to origin of the chimeric population in parous women. Similarly, in the setting of TA-MC, use of sex-based probes excludes detection of female blood donors or the study of male recipients (who are the majority of trauma patients). Furthermore, the majority of transfusion recipients receive more than one blood product, again, imparting a significant limitation in discrimination of chimeric populations.

Enhanced Methods for Detection of MC

The availability of PCR has dramatically improved sensitivity of MC detection, and was applied early to the study of TA-MC, initially through the use of semi-quantitative PCR assays. These assays employed radioactive isotope-labeled probes for detection of the PCR product with subsequent semi-quantification.[123] Although sensitive, these assays were both labor intensive and prone to PCR product contamination. In addition, the dynamic range of semi-quantification PCR systems was limited to approximately 2–3 Logs.

Development of real-time PCR-based assays vastly improved both the sensitivity and specificity of the detection and quantification of MC in humans[13, 26, 33, 124–126] and mice.[127–132] In real time PCR amplification, when a minor population of cells is spiked into a dominant host background (major population), the major host allele appears at an earlier amplification cycle number from that of the spiked minor allele. The difference in amplification cycles between the major and minor populations can be used to estimate frequency of the minor relative to the major alleles. With a limit of detection as low as one chimeric cell in a background of 10^6 host cells, real time PCR has proven effective in detecting MC in both transfused trauma patients and FM-MC. Given the greater extent of automation, these assays are less prone to PCR product contamination and much less labor intensive than the antecedent semi-quantitative assays. In addition, real-time PCR assays are quantitative with a broad dynamic range of 6–7 Logs.

Unlike the initial assays that targeted the Y-chromosome, these newer assays employ panels that combine HLA- as well as insertion/deletion (InDel) polymorphisms. These assays employ sequence-specific primers that exploit 3–4 base pair differences in the target sequences and dye intercalation to detect specific amplicons. In doing so, they achieve equivalent sensitivity and specificity as probe based systems.[133–135] This has been validated both through sequencing of reaction products as well as spiking serial dilutions of target DNA into negative DNA for the targeted polymorphism.[41] These real-time quantitative PCR assay systems are also amenable to high throughput, and high DNA input for detection of MC.

The HLA panel targets donor-recipient differences in class II genes (see figure 1). One group uses HLA-DR for this purpose, as it is highly polymorphic and has been well characterized. Although sensitive, there are limitations to using an HLA-DR panel exclusively for the detection of MC. Firstly, HLA-based techniques rely on donor-recipient mismatch and would therefore fail to detect TA-MC if the targeted HLA DR alleles are shared. Second, given the breadth of HLA-DR types, it is not feasible to incorporate the complete spectrum of HLA-DR types in the panel, and thus certain cases of TA-MC will go undetected, thereby under-representing the true frequency of TA-MC. Finally, genetic polymorphisms within the HLA class II genes may, in part, be responsible for tolerance and therefore contribute to development of MC. These concerns, therefore, motivated for introduction of a second, complementary, testing array: the InDel panel.

The InDel panel was adapted for MC analysis from earlier work conducted by Alizadeh, who focused on detection of low level engraftment in hematopoietic stem cell transplant recipients.[136] Unlike HLA-DR, InDel polymorphisms are bi-allelic loci that are unrelated to the immune response genes and distributed across somatic chromosomes, thereby eliminating linkage bias associated with HLA-based ascertainment of TA-MC. The HLA-DR and InDel assays are complementary for both the identification and quantification of MC, as evidenced by validation experiments. The combination of HLA-DR and InDel panels enables identification of an informative polymorphism in 99.5% of donor-recipient pairings.[137] The lower limit of detection for the combined HLA-DR and InDel panels is approximately 1 in 10^5 to 1 in 10^6 cells, which is largely impacted by the DNA input in the sample or the number of recipient aliquots evaluated.

In prospective TA-MC studies, pre-transfusion samples are collected first to identify the major alleles of transfusion recipients by typing the recipient using the combined InDel and HLA-DR panels. Any additional alleles that are subsequently detected in the post-transfusion sample are thereby assumed to be of donor origin and consequently chimeric alleles. In the absence of a recipient pre-transfusion samples, or in cross-sectional studies, the post-transfusion sample is serially diluted and amplified to identify the major host alleles

through disproportionate dilution of the chimeric population, rendering the latter undetectable. Once the major allele is known, the sample is amplified again using probes that target non-host alleles.

Finally, although the real-time PCR assays are quantitative, the signals of MC are extremely low and most samples require a large DNA input in order to obtain a signal sufficiently strong for quantification. For example, if the true MC concentration is 1 in 10^5 cells, a minimum DNA input equivalent of approximately 10^6 cells is required to generate a signal that is both detectable and quantifiable.

Quantification of MC

There are different strategies for reporting MC. Some report MC as a dichotomous outcome (positive vs. negative) while others extrapolate results e.g. report the MC concentration at a given cell number e.g. 100 chimeric cells per 10^6 cells.

The knowledge that 10-fold dilutions in the sample each PCR cycle will correlate with a doubling in DNA (3.3 cycle difference), enables quantification. One can compare the amplification curve of the sample with that of a standard that has similarly been serially diluted. This comparison enables quantification to a given percentage; an early cycle threshold corresponds to a high input percentage while a later cycle threshold corresponds to a lower input percentage.

In order to establish the proportion of chimeric cells, one needs first to establish the total genomic DNA input (or amplifiable genomic DNA) for the sample (denominator). In our studies this is achieved by amplifying HLA-DQ∝, a conserved region in the major histocompatibility complex II locus, and comparing the output to that of a reporting standard, i.e. samples of known genomic DNA input e.g. 10^3, 10^4, 10^5 cells/mL. Amplification of HLA-DQ∝ also provides for a common method of MC quantification, which is to divide the copy number of the chimeric allele e.g. HLA-DR4 as established first through one of the above detection methods, and to divide this by total genomic DNA input as determined by the level of HLA-DQ∝. The chimeric population is therefore reported as a percentage of the total input. Although relatively simply to calculate, this method neglects a variable efficiency of detection with respect to the different PCR targets, thereby potentially introducing inconsistency in the results.

Once the total DNA is known, a second amplification is conducted against alleles specific to the chimeric population, which is similarly compared to a reporting standard, i.e., samples of known DNA input, e.g. 1, 10, 100 cells/mL. The reporting standard used for the chimeric population is different from that used to gauge total DNA and contains the same specific genetic sequence (HLA-DR and InDel) targeted on the chimeric population. In TA-MC, the actual donor standard is unknown and an external standard is instead employed.

MC can also be reported as genomic equivalents (GEq) although this has not been widely used in reporting of TA-MC. The use of GEq is based on a correlation between DNA input and a given cell number i.e. 1μg DNA is equal to approximately 1.5×10^5 cells. The total DNA input is therefore critical to the sensitivity of the assay. With this reporting method, if the total DNA input is low, e.g. a total DNA input equivalent to 10^4 cells, one should not report the chimeric population per 10^6 cells as this could overstate the sensitivity of the assay.

CLINICAL SIGNIFICANCE OF TA-MC

The clinical ramifications of TA-MC are still unknown. TA-MC has not been extensively investigated and the studies to date have primarily focused on trying to establish the settings in which TA-MC occurs and the immunology of trauma that uniquely place transfusion recipients at “risk”. The clinical effects therefore remain speculative, based on study of MC in other settings, specifically FM-MC.[2, 22] FM-MC has been associated with a wide array of both immune and non-immune diseases, including scleroderma, multiple sclerosis[8, 138], systemic lupus erythematosis (SLE), polymorphic eruption of pregnancy[139], autoimmune thyroiditis[140], biliary atresia[141, 142] and preeclampsia. Furthermore, the observation of histopathological overlap between scleroderma, an autoimmune disease of uncertain etiology, with GvHD, a predictable effect of MC in the setting of transplantation, spurred a novel hypothesis that links FM-MC to autoimmune disease.[143, 144]

In a large study of TA-MC by Utter et al, 163 transfused combat veterans (World War II, the Korean War, and the Vietnam War) were compared with age- and gender-matched blood donor controls.[10] 9.8% of veterans vs. 0.7% of controls (RR 14.7; 95% CI, 2.0–110) demonstrated MC 46±12 years following transfusion. Subjects were questioned regarding autoimmune disease (such as Lupus, Scleroderma, Sjögrens syndrome, Reynaud’s Syndrome, multiple sclerosis), coronary artery disease and any cancer. Extending the hypothesized link between F-MC and autoimmune disease, subjects were also questioned regarding symptoms or signs that might allude to undiagnosed autoimmune disease. Similarly, subjects were questioned about symptoms that might suggest chronic GvHD, another speculated manifestation of TA-MC. No significant difference was detected between the TA-MC vs. non-TA-MC groups in any of these outcomes. Although limited by survivor bias and self-reporting, this suggests that TA-MC does not confer adverse effect in the majority of recipients. However, the numbers of subjects were very small (16 veterans with TA-MC in the Utter study), which is limiting when investigating rare outcomes such as autoimmune disease.

All TA-MC studies to-date have been performed on whole blood, given both the suitability in evaluating MC as well as the ease of availability. As a caveat, although evaluation of MC using whole blood is logistically convenient, it may under-represent the extent of systemic involvement. Tissue is necessary if one is to understand the true distribution, physiology and downstream clinical ramifications of MC.

Transfusion Associated Graft versus Host disease

One extreme clinical manifestation of TA-MC is transfusion-associated Graft vs. Host disease (TA-GvHD), a rare complication of allogeneic blood transfusion. This results from transfusion with donor cells that are homozygous for a haplotype shared by the recipient. The chimeric donor cells go unrecognized by the host, allowing them to engraft and attack the haploidentical recipient. Unlike GvHD in hematopoietic stem cell transplant, where the marrow is donor-derived and therefore spared, TA-GvHD incites global damage and is uniformly fatal with only rare, anecdotal exceptions.[145] TA-GvHD is the principle reason for irradiation of blood products collected from family members or those to be transfused to immunosuppressed patients. While rare in the United States, the incidence can be high in populations with high ethnic homogeneity such as Japan.[146] Risk of TA-GvHD has led to routine irradiation of all blood products in Japan. Although typically a dramatic clinical presentation, there remains the possibility that TA-GvHD represents a clinical spectrum that includes more subtle changes that could go unnoticed or be ascribed to comorbid disease. For example, patients transfused for trauma may develop complications such as multiorgan failure that are readily attributable to the trauma, so that TA-MC driven TA-GvHD might not be considered. Further work is in progress to test this hypothesis.

Alloimmunization

Blood transfusion and pregnancy are both established risk factors for alloimmunization, resulting in antibody formation against red cell, platelet or granulocyte antigens in the transfusion recipient or parous female respectively. The role of TA-MC in development of alloimmunization is not yet known, but is plausible. Alloimmunization adversely affects both the eligibility for transplantation as well as the ability to procure compatible blood for transfusion, particularly in chronically transfused patients. This association is also under study both with respect to WBC and RBC alloimmunization.

Consideration for Patient Typing

TA-MC may hinder resolution of complex immunohematology work-ups of the patients that have been multiply transfused or have a positive direct antiglobulin test.[147] Conventional techniques used to establish red cell phenotyping, e.g. reticulocyte separation, are complex, time-intensive and occasionally still fail to provide resolution. Patient red cell genotyping is an emerging technology to help predict antibodies that the patient could make through generation of a genotype-informed phenotype. TA-MC, whether transient or durable, can confound results both through the laboratory's unintended serological or geno-typing of a minor population.

POSSIBLE BENEFICIAL EFFECTS OF MC

A "repair hypothesis" proposes that chimeric cells with pluripotent progenitor function can develop into parenchymal cells in response to tissue injury.[148] Fetal progenitor cells have the ability for multi-lineage differentiation with subsequent recruitment and rejuvenation following tissue injury.[4, 22] Fetal chimeric cells have been shown to be significantly more common in organs of women with certain disease processes (such as SLE) with related injury, compared to both healthy controls ($p<0.001$) and to the organs from women with SLE without injury ($p=0.036$).[148] A problem shared with the autoimmune-MC hypothesis, however, is the inability to establish causality (the chicken / egg paradigm). Although chimeric cells may serve in a reparative capacity, an alternative hypothesis is that of global cellular recruitment in the setting of inflammation in affected organs that might include non-selective recruitment of resident chimeric cells.[149]

Finally, the search for improved methods of antenatal diagnosis provided the impetus that chanced upon MC. Prenatal genetic diagnosis has relied on invasive sampling e.g. chorionic villus biopsy and amniocentesis, which incurs risk of fetal loss. If chimeric fetal cells or free DNA were reliably detectable in the peripheral maternal circulation, this would bypass the need for high-risk sampling. [14, 150] Bianchi *et al.* evaluated the ability to detect MC and aneuploidy in women carrying singleton male fetuses using a blinded FISH study. They found at least one cell with an X and Y signal in 41.4% of cases. The detection rate of at least one aneuploid cell in cases of fetal aneuploidy was 74.4% with a false-positive rate estimated to be between 0.6% and 4.1%.[150] Although, comparable to single marker prenatal serum screening, further work is needed if this is to replace or compliment existing strategies of antenatal diagnosis.[121, 150]

FUTURE STUDIES ON TA-MC

Research on TA-MC has been constrained by challenges inherent to tracing large numbers of transfused subjects for prolonged periods, the high costs of laboratory evaluation and the lack of expertise and funding given a relatively esoteric field. Ongoing areas of study include populations at risk, primarily focused on patients that share overlap with the trauma population, clinical outcomes and mechanisms.

The clinical significance and potential utility of MC remains a major interest. While unrelated to TA-MC, ongoing studies are investigating the role of FM-MC in autoimmune disease. Transfusion medicine experts can play an active role in MC research given the ability to access population and pregnancy data, necessary for these studies. For example, through linkage of blood donors with known repositories (e.g. Recipient Epidemiology and Donor Evaluation Studies), one can determine rates of MC in women with different autoimmune diseases such as SLE, RA and MS and compare these with healthy female donors matched for parity and maternal-fetal HLA compatibility. Germane to transfusion is the relationship between FM-MC and alloimmunization, and whether MC influences the development and persistence of RBC and platelet alloantibodies among parous female donors. This can be extended to similar allo-responsiveness in transfusion recipients with an evaluation of TA-MC.

The mechanisms driving TA-MC have yet to be fully elucidated. While the immune changes following trauma and blood transfusion may enable MC to occur, only a subset of transfused trauma patients develop durable TA-MC, suggesting that trauma and transfusion are necessary, but not sufficient for the development of TA-MC. Further work is needed to determine whether other factors such as extent of donor/recipient HLA matching, injury severity and variability of transfused products contribute to TA-MC, and specifically, whether they influence the type of immune response.

The mechanisms enabling transfused donor cells to evade the host immune response are still unknown. Short-term term TA-MC may be explained by the transient immune disarray following trauma; however, it is less clear how the chimeric cells continue to survive (years to decades), well after the host immune system has been restored. One explanation is that of long-term peripheral tolerance to the chimeric population, either through deletion of reactive host cells, or by induction of anergy. Given the long-term persistence, the demonstration of multiple cell lineages and the increase in cell numbers in selected patients over time, the more widely accepted explanation is that of donor stem cell engraftment, with integration into the “self” repertoire, thereby imparting central tolerance to the donor. [26, 29, 34] However, persistence of tolerance-inducing donor cells could also result from long-term survival of differentiated cells.

Finally, the functionality of chimeric cells -specifically whether they are able to participate in the normal host immune response and tissue repair- is unknown. While chimeric cells do not appear to inflict damage, further work is necessary to determine whether subtle adverse or beneficial effects are being missed. Alternatively, the chimeric cells may be anergic, rendering them incapable of any functional effect.

CONCLUSION

Chimerism research still remains at a foundational level, and further work is needed to elevate this beyond scientific curiosity. FM-MC has gained some traction with compelling hypotheses linked to autoimmune disease and repair, and clinical application to antenatal diagnostics. The mechanisms that enable FM-MC to occur also hold wide-ranging implications for immunology and tolerance. In contrast, TA-MC has received comparatively less interest with studies largely confined to delineating the clinical settings in which it occurs, and the factors that predict TA-MC. To date, durable, high-level TA-MC appears confined to patients transfused following severe traumatic injury, explained in part by the characteristic immune milieu potentiated by injury. The limited clinical consequences of TA-MC observed to date should not detract from the potential importance of TA-MC, given unexplored yet plausible links to GvHD and alloimmunization. Sighting of the mythological

chimera was once considered ominous of disaster; only through improved understanding of this phenomenon can we ensure that this does not resonate with reality.

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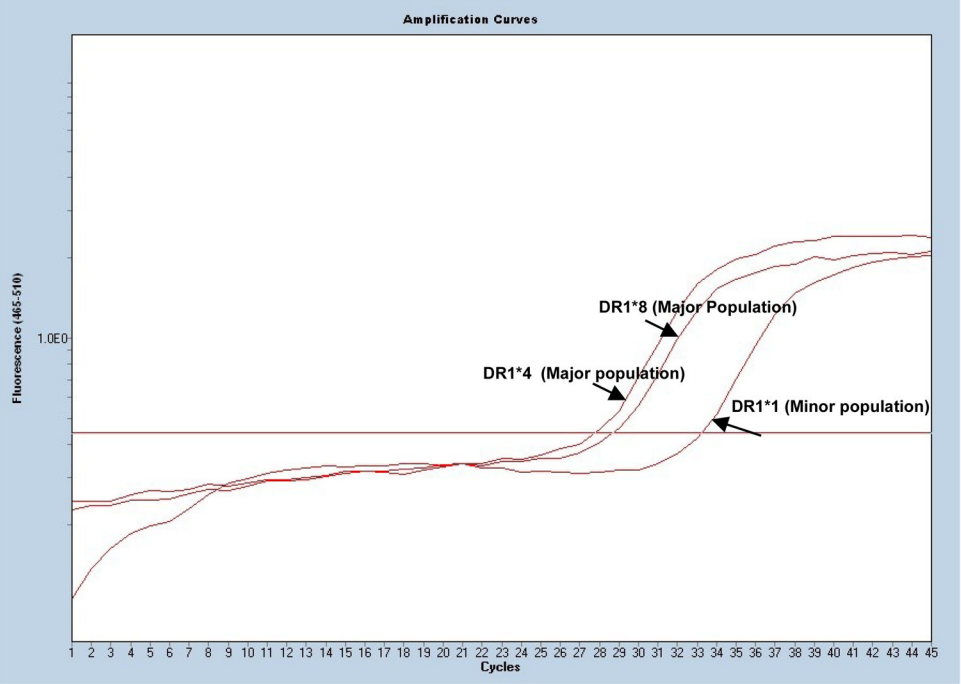

Figure 1. Real time PCR and Detection of Microchimerism

Fluorescence intensity is plotted on the y-axis while cycles are plotted on the x-axis. Cycle threshold (C_T) is defined as the cycle where the fluorescence intensity of amplified DNA crosses a background threshold. Lower cycle thresholds indicate presence of more DNA compared to samples with higher C_T s. The patient pre-transfusion sample was typed as HLA DR1*4 and HLA DR1*8, with cycle thresholds for the major types of 27.8 and 28.6 cycles, respectively. Following identification of the subject type, the post-transfusion sample was probed for microchimerism by amplifying for HLA-DR1*1, 3, 4, 7, 8, 9, 10, 11, 12,13, 15 and 16. The cycle threshold for the post-transfusion minor population of DR1*1 is 33.3 cycles.

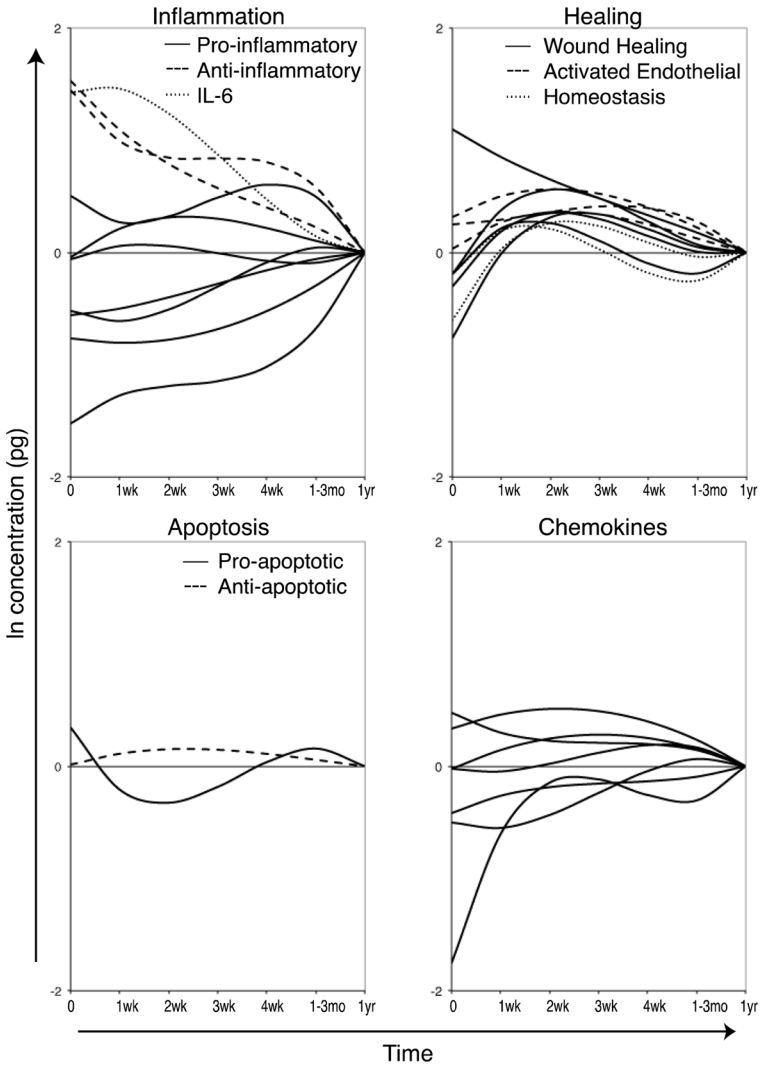

Figure 2. Kinetics of immune response to trauma

Blood samples were collected from trauma patients beginning with arrival to the emergency room and up to 1 year after injury. Multiplexing techniques were used to measure the levels of 41 immunomodulatory proteins in the plasma. Multivariable generalized estimating equations models were generated using the natural log of the concentration of each protein as the dependent variable and time since trauma, injury severity score, injury type, size of transfusion, age, sex, and microchimerism as the independent variables. Overlays of the models' prediction of the influence of time since trauma controlling for the other covariates are plotted by protein type. Predicted values at 1 year after trauma are set as the baseline (0) for each cytokine to show elevation or depression relative to this value. The inflammation plot includes the pro-inflammatory cytokines interleukin (IL)-1α, IL-5, IL-9, IL-17, tumor necrosis factor α, tumor necrosis factor β, and macrophage migration inhibitory factor, the anti-inflammatory cytokines IL-1 Receptor a and IL-10, and IL-6, which has both pro- and anti-inflammatory properties. The healing plot includes the wound healing proteins epidermal growth factor, fibroblast growth factor-2, vascular endothelial growth factor, matrix metallopeptidase 9, and total plasminogen activator inhibitor-1, the activated endothelial markers soluble E-Selectin, soluble inter-cellular adhesion molecule-1, and soluble vascular cell adhesion molecule-1, and the homeostasis cytokines IL-7 and IL-15.

The apoptosis plot includes the pro-apoptotic soluble FasL and the anti-apoptotic soluble Fas. The chemokine plot includes interferon gamma-induced protein-10, IL-8, macrophage inflammatory protein 1 α, monocyte chemotactic protein-1, eotaxin, fractalkine, and macrophage derived chemokine. (Adapted from Jackman, R. P., et al. 2012, Transfusion. doi: 10.1111/j.1537-2995.2012.03618.x, with permission)
[120]

Table 1

Techniques for detection of microchimerism

Technique	Brief Description	Advantages	Disadvantages
• Physical sorting techniques e.g. FACS * MACS * • Flow sorting	Identification of minor population using the Y-chromosome	• Ease of use • Older technique used for detection of FM-MC	• Low sensitivity • Restricted to detection of single male population (FM-MC) if using a Y-chromosome based probe
FISH *	In-situ hybridization using a labeled probe e.g. against Y-chromosome	Visual representation	• Low sensitivity • Y-chromosome based probes limit detection to single male target
PCR	Amplification of minor population e.g. using a Y-chromosome based probe	Use in both FM-MC and TA-MC	• Limited to male target (FM-MC or TA-MC) • Can't discriminate multiple blood donors • Not quantitative
Real Time-PCR using HLA-DR	Quantitative amplification of HLA-DR loci	Used for both TA-MC and FM-MC	Can under-represent MC
Real Time-PCR using both HLA-DR and InDel probes	Quantitative amplification of multiple loci (HLA and non-HLA targets)	• TA-MC and FM-MC technology higher sensitivity that HLA-DR alone • Sensitive and specific	May under-represent MC

*Abbreviations

MACS: magnetic-activated cell sorting

FACS: fluorescence-activated cell sorting

FISH: Fluorescent In-Situ Hybridization